

23 July 2010 | \$10

Science

 AAAS

EDITORIAL

- 369 Escalating Threat of Wheat Rust
Mogens Støvring Hovmøller et al.

NEWS OF THE WEEK

- 374 At Last, Vaginal Gel Scores Victory Against HIV
>> Science Express Research Article by Q. Abdool Karim et al.
- 375 Planned Study of Avandia in Doubt After FDA Review
- 376 NSF Misfires on Plan to Revamp Minority Programs
- 377 Chinese Policies Could Pinch U.S. Efforts to Make Electric Vehicles
- 377 From the *Science* Policy Blog
- 378 Last Stand on the Yangtze
- 379 From *Science's* Online Daily News Site
- 380 Nobelist Paul Nurse to Pilot Royal Society, London Superlab

NEWS FOCUS

- 382 Dams for Patagonia
A Craving for Hydropower
>> Science Podcast
- 386 Can Dogs Behaving Badly Suggest a New Way to Treat OCD?

LETTERS

- 388 Bracing for Oil
R. N. Silverstein
- Evolution of Fairness: Cultural Variability
N. Baumard et al.
- Evolution of Fairness: Rereading the Data
A. W. Delton et al.
- Response
J. Henrich et al.

- 390 CORRECTIONS AND CLARIFICATIONS

BOOKS ET AL.

- 391 Hybrid
N. Kingsbury, reviewed by A. B. Bennett
- 392 Pandora's Seed
S. Wells, reviewed by S. H. Ali

POLICY FORUM

- 393 Prepublication Data Release, Latency, and Genome Commons
J. L. Contreras

PERSPECTIVES

- 395 Launched at 36,000g
J. L. van Leeuwen
>> Brevia p. 406
- 396 Pairs Rule Quantum Interference
J. D. Franson
>> Report p. 418
- 397 Wnt Fans the Flames in Obesity
D. Y. Oh and J. M. Olefsky
>> Report p. 454
- 399 Machine Science
J. Evans and A. Rzhetsky
- 400 Carbonates and Martian Climate
R. P. Harvey
>> Report p. 421
- 402 Filtering Wildlife
J. S. Brashares
- 403 Seeing the Light of Day
C. Cepko
>> Research Article p. 413

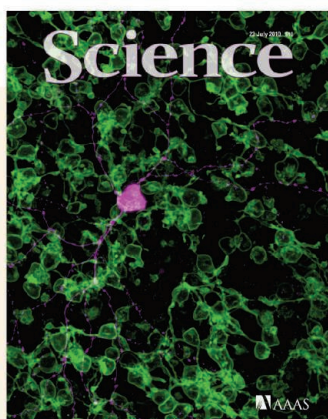
CONTENTS continued >>



page 382



page 392



COVER

Retinal photoreceptors from a mouse model of retinitis pigmentosa, a degenerative disease that leads to blindness, following gene therapy. Expression of a microbial light-activated ion pump (green) in damaged cone cells restored light sensitivity to the diseased retinas. An activated ganglion cell, which relays visual information to the brain, is shown in magenta (diameter, ~12 μ m). See page 413.

Image: Volker Busskamp and Botond Roska/Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland

DEPARTMENTS

- 367 This Week in *Science*
- 370 Editors' Choice
- 372 *Science* Staff
- 373 Random Samples
- 466 New Products
- 467 *Science* Careers

BREVIA

- 406** *Sphagnum* Moss Disperses Spores with Vortex Rings
D. L. Whitaker and J. Edwards
Fluid dynamics similar to those known to drive smoke rings shoot moss spores far and wide.
>> *Perspective p. 395; Science Podcast*
- 407** Reactivation of Hepatic EPO Synthesis in Mice After PHD Loss
Y. A. Minamishima and W. G. Kaelin Jr.
Stimulating erythropoietin production in the mouse liver points to a treatment for anemia caused by chronic kidney disease.

RESEARCH ARTICLES

- 408** Switched Magnetospheric Regulation of Pulsar Spin-Down
A. Lyne et al.
Irregularities in the pulsar rotation rates can be explained by quasi-periodic, abrupt changes in the pulsar magnetosphere.
- 413** Genetic Reactivation of Cone Photoreceptors Restores Visual Responses in Retinitis Pigmentosa
V. Busskamp et al.
A bacterial ion pump rescues visual function in damaged cone-photoreceptor cells in mouse models of retinitis pigmentosa.
>> *Perspective p. 403*

REPORTS

- 418** Ruling Out Multi-Order Interference in Quantum Mechanics
U. Sinha et al.
A multiple-slit diffraction experiment shows that interference arises from pairs of paths.
>> *Perspective p. 396*
- 421** Identification of Carbonate-Rich Outcrops on Mars by the Spirit Rover
R. V. Morris et al.
Substantial carbonate concentration in martian outcrops implies extensive aqueous activity in the past.
>> *Perspective p. 400*
- 424** Ultrahigh Porosity in Metal-Organic Frameworks
H. Furukawa et al.
The large surface areas of these materials would correspond to that of dispersed nanocubes just 3 to 6 nanometers wide.
- 428** Calcareous Nannoplankton Response to Surface-Water Acidification Around Oceanic Anoxic Event 1a
E. Erba et al.
Rather than going extinct, calcareous plankton adapted to ocean acidification ~120 million years ago.

- 432** The Landscape of *C. elegans* 3'UTRs
M. Mangone et al.
Genome-wide analysis of the nematode worm indicates a role for complex expression of 3' untranslated regions in development.

- 436** A Septin Diffusion Barrier at the Base of the Primary Cilium Maintains Ciliary Membrane Protein Distribution
Q. Hu et al.
Signaling proteins are retained in the cilium by a diffusion barrier created by a member of the septin family.

- 439** Integrative Modeling Defines the Nova Splicing-Regulatory Network and Its Combinatorial Controls
C. Zhang et al.
Network modeling reveals an RNA splicing network in the brain and uncovers complex mechanisms of regulation.

- 444** Dnmt3a-Dependent Nonpromoter DNA Methylation Facilitates Transcription of Neurogenic Genes
H. Wu et al.
DNA methylation is normally repressive but can activate genes when the methylated sites lie outside promoter regions.

- 448** Functional Modules and Structural Basis of Conformational Coupling in Mitochondrial Complex I
C. Hunte et al.
A long helix transduces conformational energy to the proton-pumping elements in complex I.

- 451** An Electronic Bus Bar Lies in the Core of Cytochrome bc₁
M. Świerczek et al.
Electrons move across the entire structure of a functional dimer of an enzyme central to cellular bioenergetics.

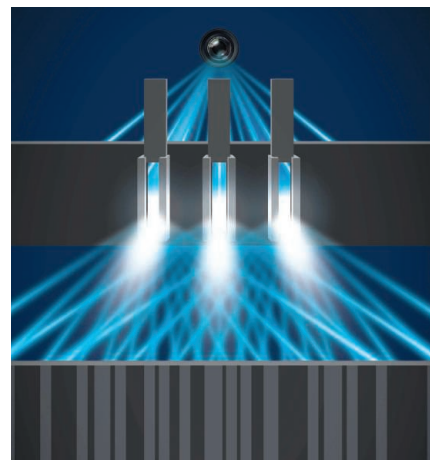
- 454** Sfrp5 Is an Anti-Inflammatory Adipokine That Modulates Metabolic Dysfunction in Obesity
N. Ouchi et al.
Fat cells secrete an anti-inflammatory factor that has beneficial effects on metabolic health.
>> *Perspective p. 397*

- 458** Muscle Dysfunction Caused by a K_{ATP} Channel Mutation in Neonatal Diabetes Is Neuronal in Origin
R. H. Clark et al.
Identification of the origin of muscle weakness that accompanies a form of neonatal diabetes may lead to safer therapies.

CONTENTS continued >>



page 399



pages 396 & 418



page 448

SCIENCEONLINE

SCIENCEEXPRESS

www.scienceexpress.org

Effectiveness and Safety of Tenofovir Gel, an Antiretroviral Microbicide, for the Prevention of HIV Infection in Women

Q. Abdool Karim et al.

Tenofovir in a vaginal gel formulation shows significant protection against HIV infection in a randomized control trial.

10.1126/science.1193748

>> *News story p. 374; Science Podcast*

The *Legionella* Effector Protein DrrA AMPylates the Membrane Traffic Regulator Rab1b

M. P. Müller et al.

An intracellular bacterial pathogen interferes with host cell membrane trafficking.

10.1126/science.1192276

Detection of C₆₀ and C₇₀ in a Young Planetary Nebula

J. Cami et al.

Hydrogen-poor conditions allow fullerenes to form in space.

10.1126/science.1192035

Decrease in the CO₂ Uptake Capacity in an Ice-Free Arctic Ocean Basin

W.-J. Cai et al.

The current carbon dioxide levels in the Arctic Ocean basin will limit further uptake under ice-free conditions.

10.1126/science.1189338

The Kamil Crater in Egypt

L. Folco et al.

An unusually well-preserved 45-meter-diameter crater provides ground truth for small-scale meteorite impacts on Earth.

10.1126/science.1190990

SCIENCENOW

www.sciencenow.org

Highlights From Our Daily News Coverage

Will IVF Work for You?

New model attempts to calculate the odds of in vitro fertilization success.

The Persistence of Memory ... in Reprogrammed Cells

Researchers can turn an adult cell embryonic, but they cannot make it forget where it came from.

The Case of the Poisoned Fuel Cell

New catalysts could revive efforts to power fuel cells with gasoline and other liquid fuels.

SCIENCE SIGNALING

www.sciencesignaling.org

The Signal Transduction Knowledge Environment

RESEARCH ARTICLE: Increased Abundance of Opioid Receptor Heteromers After Chronic Morphine Administration

A. Gupta et al.

The μ - δ opioid heteromer may be a target for alleviation of chronic pain.

LETTERS: Comment and Response on "Increased MKK4 Abundance with Replicative Senescence Is Linked to the Joint Reduction of Multiple MicroRNAs"

U. Moens et al. and M. Gorospe et al.

Questions are raised about the antibody used to detect the phosphorylated form of the kinase PRAK.

PODCAST

M. Fainzilber and A. M. VanHook

Network redundancies lend robustness to the neuronal injury response.

GLOSSARY

Find out what FAST1, ARE, and ePR mean in the world of cell signaling.

SCIENCECAREERS

www.sciencecareers.org/career_magazine

Free Career Resources for Scientists

Experimental Error

A. Ruben

Introducing the first humor column about science careers.

Re-Visioning a Career

R. Mejia

David Price launched a career in computer science after losing his vision during graduate training in geology.

SCIENCE TRANSLATIONAL MEDICINE

www.sciencetranslationalmedicine.org

Integrating Medicine and Science

PERSPECTIVE: Closing the Scientific Loop—Bridging Correlation and Causality in the Petaflop Age

G. An

High-performance computing, combined with an evolutionary paradigm for the interpretation of biomedical knowledge, can pinpoint disease causes.

PERSPECTIVE: Could Exercise Metabolomics Pave the Way for Gymnomimetics?

M. F. Burke et al.

An analysis of metabolites that change in concentration during exercise suggests possibilities for mimicking its beneficial effects.

RESEARCH ARTICLE: Comprehensive, Quantitative Mapping of T Cell Epitopes in Gluten in Celiac Disease

J. A. Tye-Din et al.

Three immunogenic peptides from gluten are mainly responsible for celiac disease, suggesting a way to replace the current burdensome therapy.



SCIENCECAREERS

Don't try this at home.

RESEARCH ARTICLE: CD4⁺CD25⁺ Regulatory T Cell Depletion Improves the Graft-Versus-Tumor Effect of Donor Lymphocytes After Allogeneic Hematopoietic Stem Cell Transplantation

S. Maury et al.

Transplanted donor lymphocytes treat recurrent malignancy more effectively when they have been depleted of inhibitory T regulatory cells.

SCIENCEPODCAST

www.sciencemag.org/multimedia/podcast

Free Weekly Show

Download the 23 July *Science* Podcast to hear about a microbicidal gel for preventing HIV infection, plans for hydropower dams in Patagonia, turbulent moss spore dispersal, and more.

SCIENCEINSIDER

news.sciencemag.org/scienceinsider

Science Policy News and Analysis

SCIENCE (ISSN 0036-8075) is published weekly on Friday, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue, NW, Washington, DC 20005. Periodicals Mail postage (publication No. 484460) paid at Washington, DC, and additional mailing offices. Copyright © 2010 by the American Association for the Advancement of Science. The title SCIENCE is a registered trademark of the AAAS. Domestic individual membership and subscription (51 issues): \$146 (\$74 allocated to subscription). Domestic institutional subscription (51 issues): \$910; Foreign postage extra: Mexico, Caribbean (surface mail) \$55; other countries (air assist delivery) \$85. First class, airmail, student, and emeritus rates on request. Canadian rates with GST available upon request, GST #1254 88122. Publications Mail Agreement Number 1069624. Printed in the U.S.A.

Change of address: Allow 4 weeks, giving old and new addresses and 8-digit account number. **Postmaster:** Send change of address to AAAS, P.O. Box 96178, Washington, DC 20090-6178. **Single-copy sales:** \$10.00 current issue, \$15.00 back issue prepaid includes surface postage; bulk rates on request. **Authorization to photocopy** material for internal or personal use under circumstances not falling within the fair use provisions of the Copyright Act is granted by AAAS to libraries and other users registered with the Copyright Clearance Center (CCC) Transactional Reporting Service, provided that \$20.00 per article is paid directly to CCC, 222 Rosewood Drive, Danvers, MA 01923. The identification code for *Science* is 0036-8075. *Science* is indexed in the *Reader's Guide to Periodical Literature* and in several specialized indexes.



ADVANCING SCIENCE. SERVING SOCIETY

Mogens Støvring
Hovmøller and
Annemarie Fejer
Justesen are senior
scientists and Stephanie
Walter is a postdoctoral
scientist in the Depart-
ment of Integrated Pest
Management, Aarhus
University, Slagelse,
Denmark. E-mail:
mogens.hovmoller@
agrsci.dk; stephanie.
walter@agrsci.dk;
annemariefejer.
justesen@agrsci.dk

Escalating Threat of Wheat Rusts

LASTMONTH, NEARLY 600 SCIENTISTS FROM MORE THAN 80 COUNTRIES CONVENED IN ST. PETERSBURG, Russia, at the International Wheat Conference to discuss the world's most widely planted crop. This came on the heels of a Borlaug Global Rust Initiative (BGRI) workshop that focused on the rapidly spreading fungal diseases known as wheat rusts, which are causing epidemics that require urgent action. If we are to prevent devastating crop losses, nations must coordinate to enact short-term solutions; they must also expand long-term efforts in research, plant breeding, and surveillance.

In the 1940s, American agronomist Norman Borlaug led the charge against wheat stem rust that threatened farmers in Mexico. By breeding new plant varieties to resist the causative pathogens, he spurred a Green Revolution that has held this worldwide threat in check since the 1970s. But in 1999, a virulent fungal strain (Ug99) was detected in Africa, making most commercial wheat varieties around the world vulnerable to stem rust once again.* While the world remains in fear of stem rust, large-scale epidemics caused by new virulent and aggressive strains of yellow rust fungus (also known as stripe rust) now pose a severe threat to the world's wheat supply. Since 2000, the United States and Australia have faced severe yellow rust epidemics. Even more alarming, last year yellow rust devastated major wheat-producing areas in China, northern and eastern Africa, western and central Asia, and the Middle East. The presence of two virulent and highly aggressive yellow rust strains (PstS1 and PstS2) at high frequencies at epidemic sites on five continents (including Europe) may represent the most rapid and expansive spread ever of an important crop pathogen. This epidemic trend may continue because the aggressive strains, which can tolerate higher temperatures, are still evolving.

Short-term responses to ongoing rust epidemics are limited to fungicide sprays, which may be unaffordable or unavailable in poor countries. Rapid replacement of the wheat most susceptible to rust with locally adapted, resistant, or less susceptible varieties is needed to slow down future spread. Any sustainable solution must involve political commitment; increased and coordinated international surveillance and monitoring; breeding for durable rust resistance; seed multiplication; and training of pathologists, breeders, and new young scientists. Fundamental research covering rust biology and epidemiology, evolutionary genetics, and rust-wheat interactions will also be required. For stem rust, such coordinated efforts have been successfully established by the BGRI and the Food and Agriculture Organization of the United Nations, which last month launched the Rust SPORE Web portal to track the advance of Ug99.† However, similar combined efforts are lacking for yellow rust, and the creation of an analogous reaction force will require substantial financial support.

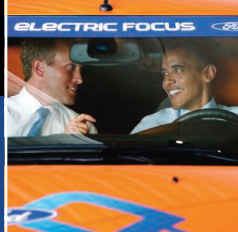
The recent agreement by the BGRI Executive Committee and international experts at the St. Petersburg meeting to establish a Global Rust Reference Center (GRRC) that targets all wheat rust fungi would unify and intensify wheat rust surveillance and training. A first step was taken last year, when the International Center for Agricultural Research in Dry Areas (ICARDA), the International Maize and Wheat Improvement Center (CIMMYT), and Aarhus University (Denmark) launched a facility to target yellow rust. The GRRC will complement existing surveillance efforts by CIMMYT, ICARDA, and national rust diagnostic laboratories and maintain a wheat rust gene bank to support resistance breeding and research. The burning question, however, is whether policy-makers in wheat-growing countries will provide the sustained support needed for national and international institutions to prepare for future challenges by the wheat rusts. Borlaug used to say, "Rust never sleeps." Events of recent years show how right he was.

— Mogens Støvring Hovmøller, Stephanie Walter, Annemarie Fejer Justesen

10.1126/science.1194925

*E. Stokstad, *Science* 324, 710 (2009). †www.fao.org/agriculture/crops/rust/stem/en/.



Power shortage
for electric cars

377

Paul Nurse
flies high

380

HIV/AIDS

At Last, Vaginal Gel Scores Victory Against HIV

Gooooo! While South Africa was in the spotlight for hosting the World Cup games, its AIDS researchers were quietly preparing for an announcement of a major milestone in their field: For the first time ever, a vaginal gel has unequivocally blocked the transmission of HIV.

In a trial that involved nearly 900 South African women, those who received a vaginal gel that contains an anti-HIV drug had a 39% lower chance of becoming infected by the virus than those who received a placebo. "It is the first time any biological intervention against HIV-1 transmission has ever shown

convincing efficacy in a large trial," says John Moore, who studies similar vaginal microbicides at the Weill Cornell Medical College in New York City. "It's a clear-cut result with obvious protection at a meaningful level."

More than 30 randomized, controlled studies of microbicides, vaccines, and drugs to date have failed to thwart sexual transmission of HIV or have yielded such marginal success that researchers wound up hotly debating the data for years after the trials were complete. But there's no ambiguity about the data from this new microbicide study reported online in *Science* this week (www.sciencemag.org/cgi/content/abstract/science.1193748) and in a presentation at the 18th International AIDS Conference in Vienna: Of the 444 women who received a placebo gel, 60 became infected with HIV versus 38 infections in the 445 women who received the microbicide. The result was statistically significant, and no serious side effects occurred. "It's a moment we've been waiting for 2 decades," says epidemiologist Quarraisha Abdool Karim, who, with her husband, Salim Abdool Karim, headed the study, known as CAPRISA 004.

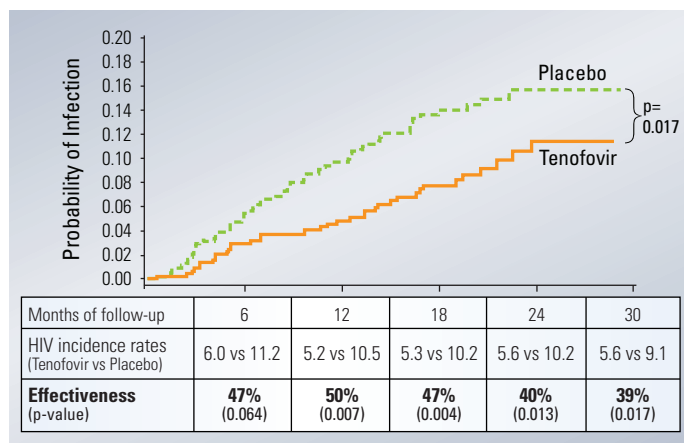
The study began in May 2007 and enrolled sexually active women between the ages of 18 and 40 who attended clinics in KwaZulu-Natal, South Africa, an area that has an extremely high rate of new HIV infections in young females. Researchers randomly assigned

the women to receive either an inert gel or the gel mixed with the anti-HIV drug tenofovir for 30 months. Participants were asked to insert the gel within 12 hours before and after having sex. They were also provided with condoms and HIV-prevention counseling.

The women reported their sexual activity each month, and the researchers also collected used gel applicators—181,340 to be exact—to monitor adherence. On average, women used the gel as advised nearly three-fourths of the time. Subset analyses showed, as expected, that the women who used the gel most frequently had the most protection: In a group of 336 "high adherers" who used the gel as advised more than 80% of the time, the reduction in risk of infection over 30 months jumped from the 39% found in the entire group to 54%. In the group that used the gel less than half the time, the risk reduction plummeted to 28%. The study also found that adherence dropped over time; among the entire group, the gel reduced the risk of infection by 50% over the first 12 months versus 39% over 30 months. "Task number one is better adherence," says Salim Abdool Karim, also an epidemiologist and the head of the Durban, South Africa-based CAPRISA, which stands for Centre for the AIDS Programme of Research in South Africa.

Both the CAPRISA investigators and other researchers who were not involved with the study stress that these results do not mean that a tenofovir microbicide gel is ready for market. "It's a really exciting first step," says Sharon Hillier, a reproductive specialist at the University of Pittsburgh in Pennsylvania, who heads the Microbicide Trials Network sponsored by the U.S. National Institutes of Health. Earlier microbicide studies had all used compounds that attacked HIV nonspecifically, using surfactants and the like, she notes. "We got a proof of concept: Topically applied antiretrovirals can interrupt HIV in women. Is it good enough? Absolutely not. We want to see something more effective." Hillier and others also say they would like to see the CAPRISA 004 results confirmed in a second study.

Hillier is heading just such a trial. Called Vaginal and Oral Interventions to Control the Epidemic (VOICE), the study is comparing two types of pre-exposure prophylaxis: daily vaginal application of a tenofovir gel and oral administration of an anti-HIV



CREDITS (TOP TO BOTTOM): COURTESY OF SALIM S. ABDOL KARIM; ADAPTED FROM S. KARIM ET AL., SCIENCE

Downloaded from www.sciencemag.org on July 22, 2010



Patagonia's
disputed dams

382



Animal models
of mental health

386

drug. (It is testing both tenofovir and a sister compound, Truvada.) VOICE, begun in September 2009, is expected to run for 4 years and enroll 5000 women in South Africa (the Abdool Karims are collaborators) and three other countries in southern Africa. "It's going to be important to have data from more than that very high incidence single site, and it's important to see if we can do better with more frequent dosing of the gel," says Hillier. Other microbicide researchers

are exploring what they believe are more potent anti-HIV drugs than tenofovir and simpler delivery methods like vaginal rings that periodically secrete the compounds.

Even if oral pre-exposure prophylaxis works, many researchers believe a microbicide gel could have many benefits for women. For one, only minimal amounts of the drug make it to the blood system, reducing the risk of side effects. Salim Abdool Karim further stresses that prevention strat-

egies, just like contraception options, are not one size fits all.

The next step for the CAPRISA researchers is to analyze why some women who received the tenofovir gel still became infected. "What undermined tenofovir's ability to protect some of those women?" asks Salim Abdool Karim. "We need to go back into the lab and understand why we didn't see a better effect to find potential avenues to try and do better."

—JON COHEN

DRUG SAFETY

Planned Study of Avandia in Doubt After FDA Review

Since 2007, controversy has swirled around whether the glucose-lowering drug Avandia increases the risk of heart attacks in diabetes patients. The effect, if real, is modest at most. And last week, a committee of 33 top diabetes experts reviewing whether the Food and Drug Administration (FDA) should pull Avandia from the U.S. market couldn't render a clear verdict, either. Twelve voted to withdraw the drug; 10 said that its use should be restricted; and the remainder wanted it to stay on the market, but perhaps with more warnings.

That decision, which left consumers and doctors hanging, also left a lingering scientific question: What should happen to a study, called TIDE, that compares Avandia and a competing drug, Actos? The double-blind, randomized study, which got under way last year, is supposed to provide what earlier, lower-quality studies don't: a clear assessment of Avandia. The drug's manufacturer, GlaxoSmithKline, supported a large, recent study that found no increased heart-attack risk. But this study, called RECORD, was not blinded, and scientists at the FDA hearing impugned both RECORD's quality and integrity. TIDE, which is meant to enroll 16,000 diabetes patients in 39 countries in a 6-year study, was designed to remedy the problem. But now it is running into trouble itself.

The principal investigator, Hertzell Gerstein, a doctor at the Population Health Research Institute at McMaster University in Hamilton, Canada, explains the challenge. Some studies suggest that Avandia reduces cardiovascular disease, and others suggest that the drug increases the risk. "If you listen to much of the evidence," he adds, "the answer

is very simple: We don't know." (Glaxo, a TIDE sponsor, approached Gerstein about doing TIDE and still provides input. But Gerstein's team collects the data and controls the analysis.) Twenty scientists on the FDA committee agreed with Gerstein, including Morris Schambelan, a doctor at the University of California, San Francisco. "Most of the people felt that, if the drug did stay on the market, we need more safety data," Schambelan says—data TIDE could provide.

But a dozen other members heard the same evidence and voted to kill TIDE because they saw it as either flawed or coming too late. One concern is about informed consent. Studies show that Actos, which has been on the market since 1999, provides the benefits of Avandia without the same risk—one reason that a few members said they felt comfortable voting to pull Avandia. But if Actos is benign, some scientists argued that TIDE amounts to a test of whether Avandia harms people—a dubious proposition. Ruth Day, who does research at the Medical Cognition Laboratory at Duke University in Durham, North Carolina, on how well people grasp informed-consent documents, said, "I do not believe that any participants in the study as currently set up will understand what they're getting into."

Another concern cited by FDA advisers is timing. William Knowler, a diabetes epidemiologist with the National Institute of Diabetes and Digestive and Kidney Diseases, said at the hearing, "Although I'd like to have the results of the TIDE study, I think at this point it's not ethical to wait another 5 or 10 or whatever years it takes to get that and leave



A bit late? Some say it would be unethical to recruit patients for a new study of Avandia.

the drug on the market." And that assumes that TIDE can sign up enough patients. So far, TIDE has enrolled 1120 people—less than one-tenth of the goal—in 23 countries.

Gerstein acknowledges that it could be difficult to complete TIDE if FDA pulls Avandia. India recently asked Glaxo to suspend TIDE in India pending FDA's decision. A Glaxo spokesperson said TIDE is moving ahead as planned. FDA declined to comment on when it might decide Avandia's fate. Regardless, worldwide sales of Avandia have already dropped by 56% since 2006.

Drug experts are being pulled in opposite directions by the Avandia case. Medicine's imperative—first, do no harm—may be at odds with the scientific tenet: When in doubt, collect more data. As FDA ponders what to do, it has issued a mandate designed to avoid similar messes in the future: All new glucose-lowering drugs must be tested in large, postmarket studies for cardiovascular risk. But that doesn't help clear up the status of Avandia—and it may not save TIDE.

—SAM KEAN

UNDERGRADUATE EDUCATION

NSF Misfires on Plan to Revamp Minority Programs

Everybody agrees that U.S. colleges and universities need to prepare more minority students to enter careers in science and engineering. But almost nobody likes a new plan by the National Science Foundation (NSF) to fold three programs aimed at achieving that goal into a still-to-be-defined initiative. Scientists and university administrators involved in the programs are up in arms, and Congress is telling NSF to go back to the drawing board.

NSF currently spends \$90 million a year on three efforts tailored to institutions that serve African Americans and American Indians: the Louis Stokes Alliance for Minority Participation (LSAMP), the Historically Black Colleges and Universities Undergraduate Program, and the Tribal Colleges and University Program. Last year, a U.S. House of Representatives spending panel told NSF to design a fourth program specifically for the nation's Hispanic population, the nation's largest underrepresented minority. That step could quadruple the pool of eligible minority-serving institutions. At the same time, as part of a larger campaign to eliminate redundancy in government, budget officials in the Obama Administration began urging NSF to streamline its stable of programs aimed at increasing the participation of underrepresented minorities in so-called STEM (science, technology, engineering, and mathematics) fields.

In an attempt to resolve the conflicting guidance, NSF requested a bit more money—\$103 million—in its 2011 budget for a new initiative that it awkwardly labeled Comprehensive Broadening Participation of Undergraduates in STEM. “We felt we had reached a plateau in college graduation rates, in Ph.D. production, and in transition to the professoriate,” says James Wyche, who headed the NSF division that runs the three minority programs before becoming provost of Howard University in Washington, D.C., earlier this year. “So the question was, do you stay the course or look for something else?”

The specific suggestion to combine programs came from the White House, he adds.

“OMB [The Office of Management and Budget] encouraged us to think about a program that would consolidate what we were doing to broaden participation among the targeted groups, something that would use the best practices from each one.”

The bare-bones budget announcement, unveiled in February, didn't provide any details. In May, NSF issued a 5-page concept paper (<http://www.nsf.gov/od/broadeningparticipation/bp.jsp>) that declared the existing programs “should serve as a foundation for a new approach,” meaning they would be dissolved. It also said that Hispanic-serving institutions, a poorly defined term for schools at which Hispanic students constitute a significant share of the overall enrollment, would be invited to seek funding. Major research universities, now partners in some existing projects run by minority-serving institutions, would be eligible to apply directly to NSF as the lead institution.

Although the paper asks for advice on how to proceed, the community had heard enough to ring the alarm. For openers, say critics, the three programs are working: Outside evaluations confirm that they are attracting and graduating more minority students. The programs have also developed an approach

that can be scaled up. So university administrators say they don't understand why NSF would want to dilute them.

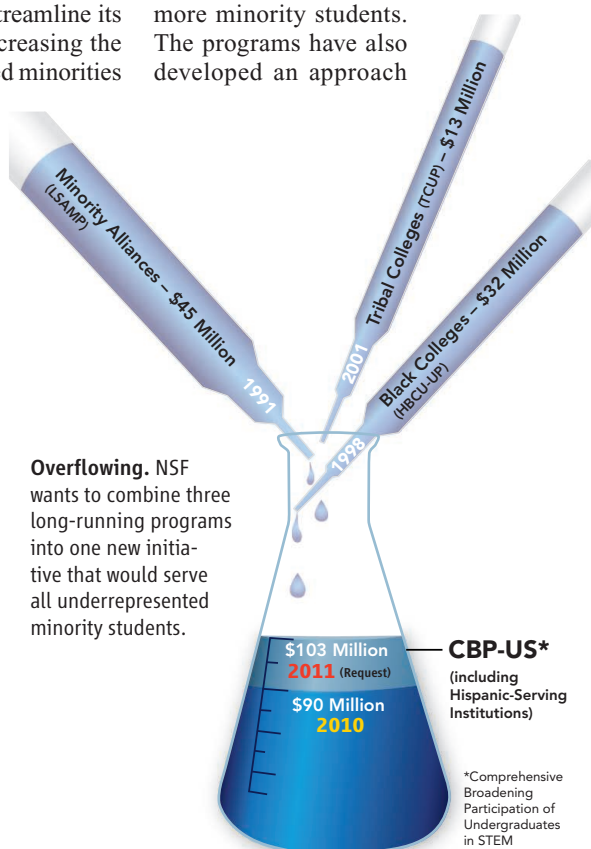
Opponents of the NSF plan also attacked the inclusion of the nation's top research universities. That change would not only open the door to institutions with a poor track record of training minorities in science and engineering fields, they said, but also give those schools an advantage because of their vastly superior resources. Finally, critics complained, NSF hadn't offered any evidence that a different approach to training minority scientists would work better.

Last month, the 42 consortia in the LSAMP program told NSF they strongly oppose the changes. Stephen Cox, provost of Drexel University in Philadelphia, Pennsylvania, and director of a project involving nine local and regional institutions, says he also wonders why NSF has put the burden for broadening participation in science on these three, relatively tiny, programs. “Rather than squeezing more blood from this stone,” Cox said, “why doesn't NSF get some more stones?”

Congress doesn't care much for NSF's new idea, either. This spring, the House of Representatives, as part of its reauthorization of the America COMPETES Act that covers NSF's research and education activities (H.R. 5116), told NSF to keep the three programs intact in 2011 and submit a report “clarifying the objectives and rationale for such changes” before heading off in a new direction. “It's something they had not discussed with us,” says Representative Eddie Bernice Johnson (D-TX), a senior member of the House science committee, who crafted the language on the minority programs. “I think they need to discuss it with stakeholders, who told me that it seems like a way to cut a lot of money from these programs.” A Senate version of the reauthorization bill introduced last week (S. 3605) would also preserve the programs, and a House spending bill for 2011 likewise tells NSF to keep funding them.

NSF appears to be rethinking its strategy. Joan Ferrini-Mundy, acting head of NSF's education directorate, which oversees these programs, says NSF is looking hard “at what a transition would actually look like. A lot is still on the table.” She also notes that “broadening participation is an NSF-wide commitment” and that any final plan is likely to involve the agency's six research directorates as well.

—JEFFREY MERVIS



RARE-EARTH ELEMENTS

Chinese Policies Could Pinch U.S. Efforts to Make Electric Vehicles

Last week, President Barack Obama highlighted his commitment to clean-energy jobs by visiting the last of nine battery-manufacturing plants to be funded from last year's massive economic stimulus package. Speaking at a groundbreaking ceremony for a Holland, Michigan, company that will build lithium-ion batteries for future electric cars and gas-electric hybrids such as the Chevy Volt, Obama declared that the plant "is a symbol of where Michigan is going, ... of where Holland is going, ... of where America is going."

That is, unless it runs into a Chinese brick wall.

This month, China announced that it will cut exports this year of rare-earth elements (REE) by 40%, leaving demand outside China exceeding the supply for the first time ever. Combined with Chinese export tariffs of 10% to 25%, the policy could ground fledgling efforts to build clean-energy industries in the United States and other Western countries. "It will just be untenable to compete" with companies based in China, says Jeffrey Green, president of J. A. Green and Co., a government-relations firm in Washington, D.C., specializing in rare earths.

China currently produces more than 97% of all rare earths, a group of 17 elements consisting of scandium, yttrium, and the 15 lanthanides. They are vital for a host of electronics and green-energy technologies, and their use is expected to triple between 2000 and 2014, topping 200,000 metric tons. But despite rising global demand, China has focused increasingly on domestic needs.

In response, the United States and other countries are gearing up for production. Molycorp Minerals in Greenwood Village, Colorado, for example, is expected to reopen its mine in Mountain Pass, California, in 2012. But that mine's annual output of 20,000 metric tons a year won't fill the gap left by lower Chinese exports.

That gap could spell trouble for the Obama Administration's plans to develop electric vehicles. In recent years, the U.S. Department of Energy (DOE) has spent roughly

\$5 billion on projects to promote electric-vehicle technologies, including nine battery-manufacturing plants and 11 electric drive component-manufacturing facilities. Last week, DOE said that by 2015 these investments would give the country the capacity to produce up to 40% of all advanced batteries manufactured globally for electric vehicles. Last year, the U.S. share was 2%.

Most of the new advanced batteries are



Road bump? President Obama's efforts to promote electric-vehicle production may be stymied by getting access to rare-earth elements.

slated to be lithium-ion batteries, which do not require rare earths from China. Even so, lithium is mined in only a few countries, which has also prompted concerns about supply shortages. And current hybrid-car batteries typically include more than 10 kilograms of lanthanum, the lightest of the rare earths.

Although one of the most abundant rare earths, lanthanum could be hardest hit by China's new export controls, which cap overall exports. Observers worry that companies, to increase profits, may try to export more high-value REEs, such as dysprosium and terbium, and drastically reduce lower-value elements such as lanthanum. That change, in turn, could result in price hikes for some elements.

To counter the advantages enjoyed by Chinese companies, U.S. companies that make magnets and other high-tech components want Congress to set up loan guarantees to back domestic mining, processing, refining, purification, and metals production of rare earths. Bills have been introduced in both houses, but no action is expected in the current session.

—ROBERT F. SERVICE

Science Insider

From the Science Policy Blog



Climate scientist and activist **Stephen Schneider**, 65, died 19 July of an apparent heart attack. His death ends a nearly 40-year career doing climate science, assessing climate science for policymakers, explaining it articulately to the public, and defending it energetically against skeptics. <http://bit.ly/stephen-schneider>

A spending panel in the U.S. House of Representatives voted to give **DOE's Office of Science** next year only what it received this year, erasing a \$221 million increase requested by the Obama Administration. The **National Institutes of Health** did a bit better from a sister panel, receiving its full request: a \$1 billion bump from its current \$31 billion budget. <http://bit.ly/2011DOE-mark>

A federal judge has dismissed charges against **four animal-rights activists** accused of harassing researchers at two University of California campuses in 2007 and 2008. But prosecutors could file new charges. <http://bit.ly/charges-dismissed>

A provocative new analysis suggests that **illegal logging** has declined 22% worldwide since 2002, thanks to stricter government policies and enforcement. Some experts are skeptical of its conclusions, however. <http://bit.ly/less-logging>

More than 2200 Russian researchers have petitioned the government asking it to **consult with the scientific community** when making major science-policy decisions. <http://bit.ly/russian-letter>

The Royal Society and the British Academy have strongly warned the British government that **looming cuts to science funding** could be "irreversibly catastrophic for the future of U.K. science and economic growth." <http://bit.ly/ukbudget-advice>

A committee of the National Research Council has provided the latest and most quantitative estimates yet of **how the coming global warming could affect the world**. The picture hasn't gotten any prettier. <http://bit.ly/climate-stabilization>

For more science policy news, visit news.sciencemag.org/scienceinsider.



Fading pulse. IHB keeps a few finless porpoises in captivity, but a 10-year ban on Yangtze fishing may be their only hope of survival in the wild, argues Wang Ding.

CONSERVATION BIOLOGY

Last Stand on the Yangtze

SHISHOU, CHINA—A hundred meters or so off the bow of our motorboat, a dark shape breaks the surface and quickly vanishes. Was it a trick of the light? Heavy rains have washed nutrients into the lake, turning the usually clearer waters emerald green. A few minutes pass, then ecologist Xie Songguang, perched like a coxswain, points off starboard and cries: “Over there!” The earlier sighting was no mirage: Two more glistening humps slice through the waves.

The Yangtze finless porpoises in Tian-E-Zhou Oxbow Nature Reserve, near the town of Shishou, dare not approach; too many of their kind have perished in encounters with humans. The world’s only freshwater porpoise is down to fewer than 1800 individuals, less than half the estimated population a decade ago. A few dozen porpoises live under the watchful gaze of rangers in Tian-E-Zhou, an oxbow lake that once was a 21-kilometer-long section of the Yangtze River. But time is running out: Unless threats to its survival are met head on, the porpoise could be gone in 15 years, says Wang Ding, an ecologist at the Institute of Hydrobiology (IHB) in Wuhan.

The finless porpoise is an icon of an ecosystem under siege. The Yangtze, the world’s third longest river, faces manifold threats, including overfishing, heavy boat traffic, and habitat fragmentation due to hydropower projects such as the mammoth Three Gorges Dam (*Science*, 1 August 2008, p. 628). Two charismatic animals—the Yangtze river dolphin, or *baiji*, and the Chinese paddlefish, a titan that once reached 7 meters in length—are down to a handful of individuals or have already gone extinct.

The loss of such top carnivores is an early sign of ecosystem collapse, says David Dudgeon, an aquatic ecologist at the University of Hong Kong. Adding insult to injury,

Yangtze fishing communities have already begun to forget that these creatures even existed, Samuel Turvey of the Institute of Zoology in London and colleagues report in the June issue of *Conservation Biology*. “I was extremely surprised that local ‘community memory’ of the Yangtze megafauna is being lost so rapidly,” Turvey says.

Researchers hope to make a last-ditch effort to stabilize the Yangtze ecosystem and prevent further extinctions. IHB is working with China Three Gorges Dam Corp. to set up a reserve in the dam’s reservoir, and Honghu Xin-Luo National Baiji Reserve plans to launch a finless porpoise *ex situ* conservation program. Meanwhile, influential researchers are calling for a lengthy fishing moratorium for the entire Yangtze. “If we can’t control overfishing, all other approaches will fail,” says IHB’s Xie.

Troubled waters

The IHB researchers know all too well what it’s like to watch a species fade into oblivion. In 1978, IHB ecologist Chen Peixun was tapped to form a *baiji* study team. Within months, the team deduced that the species was in trouble—“and that it would go extinct if we didn’t do anything,” Chen says. She puts the blame squarely on human activity. During the 1990s, about 40% of known *baiji* deaths were caused by illegal electric fishing gear. Many animals also died from run-ins with ships, and others were killed when sandbars—a favorite hangout for cetaceans—were blasted for easier navigation.

IHB researchers gleaned what they could from a captive male *baiji* named Qiqi. The aim was to understand the creature well enough to transfer rescued animals to Tian-E-Zhou, which in 1992 was designated a national preserve for *baiji* and finless porpoises. But it

was too late for the *baiji*. Yangtze surveys charted a decline from 11 sightings in 1997 to two in 1999. Then in 2002, Qiqi died of old age, apparently. That was the last *baiji* Chen and other scientists laid eyes on. In 2006, not a single *baiji* was spotted in an exhaustive survey, making it the

first human-caused extinction of a cetacean (*Science*, 22 December 2006, p. 1860).

Several measures may avert a similar fate for the Yangtze porpoise. More sanctuaries could help, for starters. The porpoises reproduce readily in Tian-E-Zhou: In 2008, all five mature females there gave birth. But small reserves can become a death trap. In early 2008, unprecedented in recent memory, much of Tian-E-Zhou’s surface froze over for 2 days. Five porpoises asphyxiated, including three pregnant females. “If the ice had lasted much longer, all the porpoises would have died,” says IHB’s Hao Yujiang.

Food supply is another constant worry. Tian-E-Zhou is 250 kilometers east of Three Gorges Dam. Before 2003, when the reservoir behind the dam began to fill, the lake’s connection to the Yangtze lasted the whole rainy season, from May to October. Now, that flow occurs only during the rainiest months of June and July. A too-brief connection suppresses fish spawning, says Hao, so researchers must stock Tian-E-Zhou with fish for the porpoises to eat.

Elsewhere, the main threats to the porpoise are overfishing, which reduces food supply, and the illegal use of nets slung across the river, which ensnare the mammals. Yangtze fish stocks have plunged since the 1950s, possibly limiting the porpoise’s recovery, says Dudgeon. The key to survival may be a blanket fishing moratorium. At a workshop in Chongqing last September, IHB’s Cao Wenxuan, one of China’s most esteemed fish specialists, called for a 10-year fishing ban for the whole Yangtze. That would be feasible, Wang argues, because the 100,000 tons of fish hauled from the Yangtze each year is less than 1% of China’s freshwater production, including aquaculture.

There is no sign that authorities are ready to take such a step in the near future. That riles Dudgeon, who penned a requiem for Yangtze ecology in the March/April issue of *Aquatic Conservation*. “How many icons do we need to lose?” he asks.

—RICHARD STONE

From *Science's* Online Daily News Site

Will IVF Work for You?

For couples who have trouble conceiving, in vitro fertilization (IVF) is often the last resort. Now researchers say they have come up with a way to better quantify a couple's chance of having a child using IVF.

A woman's age is an important factor in determining whether a couple will become pregnant, but it's far from the only one. So Mylene Yao of Stanford University School of Medicine in Palo Alto, California, and colleagues collected data on 20 variables that influence the success of IVF. These included age, number of previous pregnancies, and sperm count, and figures known after the completion of the first round of IVF, such as whether there was fertilization, endometrial thickness, and average number of cells per embryo. In all, the team fed data on more than 1600 first rounds of IVF into a computer model called a boosted tree.

When the researchers compared the model's predictive powers with a model based on age alone, they found that it was significantly more accurate in gauging success in the second round of IVF, they reported



in the *Proceedings of the National Academy of Sciences*. In the future, such a model could help women decide whether to continue using IVF or whether to move on, experts say. <http://bit.ly/IVF-chances>

Why Gorillas Play Tag

A new study of gorillas at play indicates that the animals know the limits of their social status—and that they play tag to help even the score.

Behavioral biologist Marina Davila Ross of the University of Portsmouth in Hampshire, U.K., and colleagues watched videos collected over 3 years of gorillas at various zoos and reserves in Europe. During play, some gorillas hit their playmates and ran away. Ross's team noticed a pattern in this game of tag: Gorillas lower on the social ladder were usually the taggers. These gorillas were also twice as likely to instigate another round of the game, and they frequently bared their teeth—a possible indication that they were willing to bite the other gorilla, the team reported in *Biology Letters*.

The findings, Ross says, suggest that low-status gorillas use the game as a sort of ego

boost. And that means that gorillas are aware of inequities in their society, Ross says, marking the first time that such cognition has been observed in gorillas in a nonexperimental setting. <http://bit.ly/gorilla-tag>

A Jupiter-Sized 'Comet'

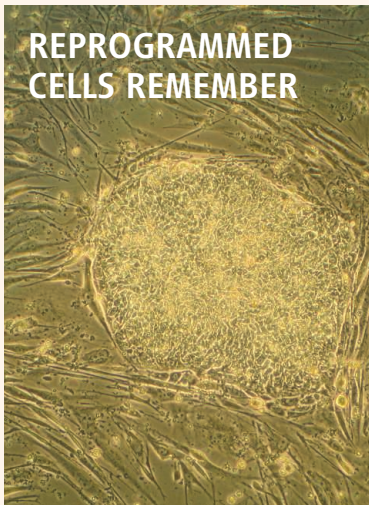
Comets aren't the only celestial bodies with long, glowing tails. A star 153 light-years from Earth is blowing away the atmosphere of planet HD 209458b, creating more or less the same effect. According to observations by NASA's Hubble Space Telescope reported this month in *The Astrophysical Journal*, the gas giant circles so close to its sun (an orbit of 3.5 days compared with Mercury's 88) that solar winds are slowly roasting it out of existence, causing its outer gases to evaporate into space. The planet, which is only slightly smaller than Jupiter, isn't



going to disappear anytime soon, however: Researchers estimate it will take a trillion years before HD 209458b is completely vaporized. That's about 100 times as long as the universe has been around. <http://bit.ly/planet-comet>

Read the full postings, comments, and more at news.sciencemag.org/sciencenow.

REPROGRAMMED CELLS REMEMBER



Like a Texan who keeps his drawl after moving to California, induced pluripotent stem (iPS) cells—adult cells reprogrammed to resemble embryonic cells—retain signatures of the tissue from which they came, according to two new studies.

George Daley of Children's Hospital in Boston and colleagues tried to make blood from different types of iPS cells. iPS cells derived from connective tissue cells didn't work, but ones reprogrammed from blood cells made plenty of blood. The reason may be that each iPS cell line sports DNA methylation, a molecular coating on the DNA that turns genes on or off, that is typical of the cell type it came from, the team reported in *Nature*.

In a related paper in *Nature Biotechnology*, Konrad Hochedlinger of the Harvard Stem Cell Institute and his colleagues found that they could identify the origin of different iPS cell lines by simply looking at their gene expression. The differences gradually disappeared as the stem cells continued to replicate in the lab, so reprogramming adult cells seems to be a gradual process, Hochedlinger says. Both studies add to the growing evidence that reprogrammed cells are not exactly like traditional stem cells derived from embryos. <http://bit.ly/cell-memory>

NEWSMAKER INTERVIEW

Nobelist Paul Nurse to Pilot Royal Society, London Superlab

NEW YORK CITY, NEW YORK—In the late 1980s, working on yeast genetics experiments that would lead to a Nobel Prize, researchers in Paul Nurse's lab in Oxford, U.K., faced an unusual initiation rite: a ride in a two-seater glider piloted by their boss. The trip usually involved Nurse turning the dual controls over to his passenger, recalls his former postdoc Christopher Norbury, now a molecular biologist at the University of Oxford. "I loved it," but, Norbury tactfully notes, "a small minority didn't enjoy it so much." Nurse himself seemed to fear nothing, Norbury says.

Nurse, now the president of Rockefeller University in Manhattan, still flies; his latest outings are in a 1934 Boeing Stearman biplane with an open cockpit. And his fearlessness apparently remains intact, as he has recently agreed to leave Rockefeller to take on two of the highest-profile jobs in British science. Earlier this month, he was confirmed as president elect of the Royal Society, the U.K. scientific society founded 350 years ago. And last week, Nurse took on another challenge, agreeing to be the founding director and chief executive of the UK Centre for Medical Research and Innovation (UKCMRI). When it is up and running 5 years from now with 1500 researchers and staff members, it will be the largest research institution in Europe.

Leszek Borysiewicz, chief executive of the U.K. Medical Research Council, which is funding the majority of UKCMRI, calls the founding director position "perhaps the most important [science] job in Europe." And it's one that will need a steady pilot, given that UKCMRI is being born out of a controversial decision to relocate the MRC's legendary National Institute for Medical Research (NIMR) from its suburban Mill Hill campus outside London to the city center (*Science*, 14 December 2007, p. 1704). Many onlookers consider the NIMR move—which merges resources from Cancer Research UK, the Wellcome Trust, and University College London—a big gamble. It will tie up £600 million, or nearly a billion U.S. dollars, at a time when British science may be facing the toughest financial crisis in 2 decades.

For the past 2 years, Nurse has chaired the UKCMRI scientific planning committee, an appointment respected even by those critical of the decision to close NIMR. And although he has outlined a vision for the planned lab, many details remain unclear, including how many NIMR scientists

will make the move to London (*Science*, 11 December 2009, p. 1468).

Although Nurse, 61, was an obvious candidate to become the facility's first director, he originally told some people that he wasn't interested. He says he changed his mind when he learned about 6 months ago that the Royal Society planned to nominate him as president. The 5-year presidency is unpaid and only half-time; the UKCMRI directorship, a 5-year planning position, will also be half-time. "I did need another position" besides heading the society, he explains, and the two jobs make for "a complete package." He becomes Royal Society president on 1 December and joins UKCMRI on 1 January.

The twin appointments are the capstone to a career full of unlikely twists. Nurse was born into a working-class family; his father



Mixing bowl. The planned UKCMRI lab in London will blend scientists from several organizations.

Q: Can you describe your vision for UKCMRI?

P.N.: One reason size is important is to take a multidisciplinary approach. Because it's large, it doesn't actually have to have a particular focus. If you set up an institute with 100 to 150 people working on stem cells or RNAi, the problem is, it often goes stale.

I've thought a lot about how to have an institute that will move with the times. About two-thirds of the 120 research groups will be at the junior end, in their 30s to early 40s. When they're developed, we will export them, help them find positions elsewhere. This is completely different from the usual philosophy in the U.S. where institutions try to hang on to people. It's a very supportive role for the whole national endeavor.

The second thing is that it won't be divided into academic departments and divisions. It's self-assembled from below.

Individuals can belong to several interest groups and if their interests change, they can withdraw from one or join another.

Q: Can you clarify your recent comment about funding scientific elites?

P.N.: I think I was misunderstood. Some people thought I was going to kill all research funding for everybody except for 100 people, which is obviously stupid. All I was saying is that for the very best, we might want to think of supporting them in a nonbureaucratic way which gives them the maximum time for their creativity.

I was very much impressed by the way Howard Hughes [Medical Institute] does funding—I'm a Howard Hughes trustee—and I thought that would be a very interesting way of doing things not simply in biomedicine but in physical sciences, chemistry, maths. [Selected researchers] would be reviewed every 5, 6, 7 years, and if they're

still highly productive, they get another 7-year tranche.

Most of these individuals are already getting research council funding and so on, and it's just a question of repackaging it in a different sort of way. It would be a couple of percent of the total funding of research. It could be administered by the Royal Society. This has got to be discussed. This is just me floating an idea.

Q: What are your thoughts about the possible U.K. science budget cuts?

P.N.: Two things. We have to tell the government that by cutting research today, they are in danger of burning the seed corn of the future. Because it's out of science that we will get the engine of wealth creation and improving health and improving the quality of life and our environment.

The second thing is that if they are going to reduce spending, they must always think

was a mechanic, his mother cleaned houses in northwest London. There were “hardly any books at home,” he says, yet he became fascinated with science by the age of 8, when he watched the Sputnik 2 spacecraft flying above London. After secondary school, he was initially rejected from universities because of the language requirement—he flunked a French exam several times. Luckily, a professor negotiated an exception.

After graduate school in biochemistry, he switched to yeast genetics and cell biology. Later at the London labs of the Imperial Cancer Research Fund (ICRF), a U.K. charity, and at the University of Oxford, his group discovered an enzyme that controls yeast cell division, known as a cyclin-dependent kinase, and showed that humans share the same gene for this enzyme. Because cancer involves uncontrolled cell division, the finding had implications for understanding human cancer. The work garnered Nurse the 2001 Nobel Prize in physiology or medicine with Tim Hunt and Leland Hartwell.

Although he’s never stopped doing research, Nurse became head of ICRF in 1996 and oversaw a sensitive merger with another cancer charity to create today’s Cancer Research UK. In 2003, after the merger, he relocated across the Atlantic to become president of Rockefeller. There “he really changed the atmosphere in a number of ways,” says Princeton University molecular biolo-

gist David Botstein, a member of the Rockefeller board: Nurse made decision-making more transparent and inclusive of junior faculty members. He also recruited new faculty based primarily on their qualifications and not because they worked in a particular discipline, an uncommon approach, Botstein says.

Already a highly visible public figure in Britain, where he is a regular guest on the BBC (*The Sun* once called him “the David Beckham of science”), Nurse has also worked to explain science to a broad U.S. audience as a guest and co-host on *The Charlie Rose Show*.



Anarchist. Paul Nurse wants to break down barriers separating biomedical researchers. ▶

about continuing to support the quality work. I wouldn’t recommend them trying to second-guess the areas because usually committees don’t do that well. What I would emphasize is supporting the highest quality people.

Q: What do you think of the push for more translational medicine research?

P.N.: When I was in the U.K., we used to think that everybody in the U.S. knew how to do translation. Now when I’ve come to the U.S., I find that everybody is worried themselves about how they do translation. I’m beginning to think that this is something nobody has really got on top of properly.

This is worth really looking at freshly, how to do things. This new institute will look at that. I’m going to start by focus-

ing on the different cultures of the people involved. Because we have basic scientists, we have clinicians, we have the pharmaceutical industry. I’m by no means certain that we’ve worked hard enough on the sociology of that, to get that to work well.

Q: Why did you decide to share your birth story with the public?

P.N.: For my [birth] mother, actually. She had had to keep this secret for half a century because of the shame of it. And I felt by talking about it in public, it somehow in part compensates for that. It may sound a

Four years ago, his life took another unexpected turn. In the course of applying for a U.S. green card, which required obtaining the long form of his U.K. birth certificate, Nurse came to realize that the people he thought were his parents were his grandparents and that his much older “sister” had given birth to him out of wedlock at age 19. None of the three is alive, and Nurse does not know who his father was. He has willingly shared the poignant story with the public.

Nurse’s current home, the leafy, gated campus of Rockefeller, feels like a serene oasis next to the chaos of Manhattan’s streets. But Nurse proudly suggests that “anarchy” exists under the surface. He likes the university’s lack of traditional academic departments, which he believes impede collaborations. He plans to bring this strategy to UKCMRI. “We’re mixed up. And that’s a little bit of what I’m trying to achieve in the other place,” Nurse says.

On a steamy summer afternoon last week, the same day his UKCMRI appointment was announced, the white-haired scientist, dressed casually in a white shirt and gray slacks, spoke with *Science* about his past, his recent suggestion to bolster U.K. funding for 100 to 150 top researchers, and his busy future. His remarks have been edited for clarity.

—JOCELYN KAISER

bit strange psychologically, but I’m saying, “No, this is OK, I’m talking about it publicly because it wasn’t such a big shame.”

There’s something ironical about being a really good geneticist and having my own genetics completely secret for so long. The irony almost appeals to me.

Q: What will you do with your lab?

P.N.: I will keep it for the next year or two then later will establish a lab in London. To keep a lab going is what keeps me sane and close to graduate students and postdocs. I never seem to do anything quite properly anymore because I’m juggling so much. But I sort of keep afloat. I wouldn’t want to just run something without having my own scholarly activity. I sometimes think the people who just want to run things are not always the right people to run them.

Dams for Patagonia

Pressed by a demand for electricity, Chile is considering seven big dams and a transmission line through its southern wilderness; critics say the environmental risks have not been fully examined

COYHAIQUE, CHILE—In rolling hills at the foot of a basalt massif, the people of this compact, ordered town live mainly by fishing and cattle ranching. For many, life is not dramatically different from that experienced by the pioneers who first cleared the valley nearly a century ago and built timber homes. But graffiti around town reveal a new disquiet. “Patagonia Sin Represas!” (“Patagonia Without Dams!”) is perhaps the politest of the slogans sprayed across the walls and buildings of this place, the capital of the Aysén region in Patagonia. They reflect anger over plans to build at least seven major hydropower dams in the area.

Home to condors and alpaca-like guanacos, puma, and blue whales, Patagonia is the tail end of the Americas, one of the last accessible nowhere lands on the planet. It contains the Southern Ice Field, the world’s third most important reserve of freshwater after Antarctica and Greenland. And in its untamed wilderness of glaciers and mountain peaks, companies are preparing to raise not just hydrodams but also a 70-meter-high transmission line to transport power more than 2400 kilometers north to Santiago, Chile’s capital, and the energy-hungry mines beyond. The line would require one of the world’s biggest clear-cuts, a 120-meter-wide corridor through ancient forests—fragmenting ecosystems—

and the installation of more than 5000 transmission towers.

Proponents of the dams argue that hydroelectricity is a clean source of energy, that Chile needs the 3500 MW/yr of power to meet its development goals and, lacking oil or coal reserves, has no viable alternative (see sidebar, p. 384). But more than 50 international environmental groups have come together to try to block dam construction under the umbrella organization that uses the slogan “Patagonia Sin Represas” as its name.

“People feel strongly about these dams,” says Peter Hartmann, regional head of Chilean Friends of the Earth, one of the main opposition groups. “The megadam projects would change this region radically and ruin the valleys.” So unpopular are the construction schemes that Chile’s second biggest bank, BBVA, announced in January that it would not be assisting the power company, HidroAysén, with loans for hydroprojects, citing environmental and social concerns.

The controversy raises questions about the goals of economic development and about the definition of environmentally clean energy—issues that divide the entire nation. National surveys show that about 53% to 57% of respondents are against the dams. This ensures that the government,

Hydroelectric gold. Patagonia’s glaciers hold one of the planet’s largest freshwater reserves.

which in the coming months is supposed to rule on the viability of initial projects, will face trouble no matter what it decides. Questions have been asked and answered by the thousands; still, key information is lacking.

Unquiet land

In August 2008, HidroAysén, the company behind five of the proposed dams, submitted its environmental impact assessment (EIA) to Chile’s national environmental authority CONAMA for regulatory approval. It is one of 32 government departments charged with assessing the EIA; reviewers found the document so wanting that CONAMA instructed

the company to address more than 3000 comments and gave a 9-month extension.

In October 2009, HidroAysén submitted its response, a 5000-page document that brought more critical comments. These included criticisms that the EIA lacked data on seismic risks in an area known for earthquakes and volcanoes; gave no accounting of glacial lake outburst floods (GLOFs); and had insufficient information on impacts to key natural habitats, biosphere reserves of

Online

sciencemag.org



Podcast interview
with author

Gaia Vince.

global importance, and wetlands and aquifers. The departments issued another 1000 comments in January 2010; HidroAysén's response—after another extension—is due in October.

According to Hartmann and his supporters, the EIAs submitted so far have overlooked entire areas of impact. “Many of the places to be assessed are inaccessible without a helicopter, so the assessors fly in, stay one night and see no wildlife and conclude there's nothing there. But that's no way to study an area,” Hartmann says. Among the diverse species threatened by the dams, he says, are endangered huemul deer (the Chilean national symbol), native fish otter, and unique cold-water corals that are only now being discovered by marine biologists at river outlets.

One of the three dam sites proposed by the company XSTRATA on the river Cuervo lies directly above the Liquiñe-Ofqui fault that runs northward from a triple junction where the Nazca, South American, and Antarctic plates meet. The plates move relatively slowly here, about 2 cm per year, compared with farther north near Concepción where the rate averages 10 cm per year, and the friction is less, says geologist Fabien Bourlon of the Research Center of Patagonian Ecosystems (CIEP) in Coyhaique. That means that the likelihood of a massive magnitude-8.8 earthquake, as occurred at Concepción in February, is less, but nevertheless, there is no study to support this, he adds.

Chile's National Geology and Minerals Service agrees, saying that proper seismic studies need to be carried out before a hydrodam is approved at the site. In 2007, 1 month after XSTRATA submitted its EIA declaring the Cuervo siting to be on a seismically inactive zone, the area experienced a massive earthquake that dislodged boulders into the fjord below, triggering a tidal wave that killed people on the opposite bank. “The government threw out their report,” Hartmann laughs.

“Actually, what we're seeing at the point where the three plates meet is an opening process—a rift—so volcanic or magmatic material could rise there,” Bourlon says.

“And there's a volcanic gap at the dam sites. A line of volcanoes stops north of the site at Chaltén, and then there are none until Hudson to the south. So it's likely there's an undiscovered volcano there,” he adds, maybe smack in between at Mount Arenales. “This area is very little studied because it's between two ice fields.”

Known volcanoes could be serious trouble for a dam, too, Bourlon says. If Hudson were to erupt, large pieces of rock and debris could rupture the turbines, and volcanic ash could quickly silt it up. No one has looked at these risks, he adds. The transmission line for the project was originally planned to run right over Chaitén, the volcano that erupted so spectacularly in May 2008, with plumes of ash that rose 16,700 meters and buried the village. HidroAysén now plans to reroute the line, most probably via the coast.

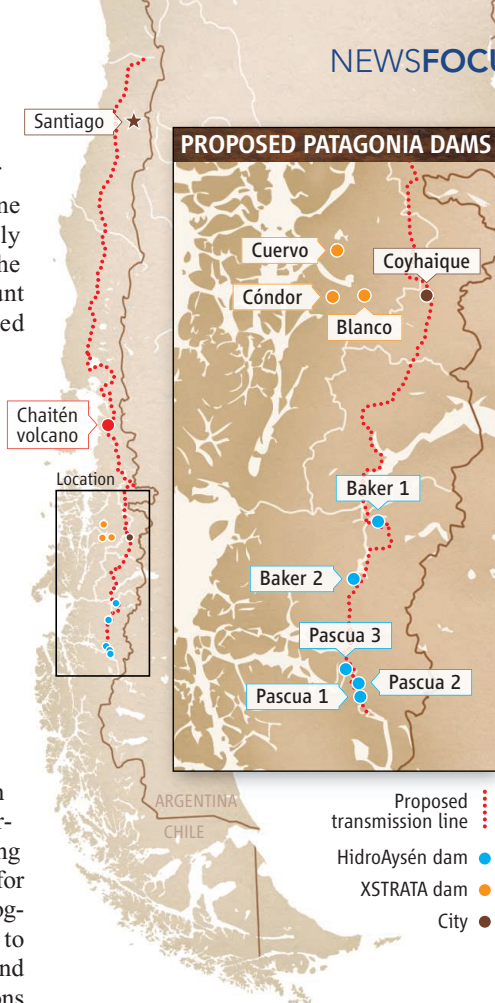
XSTRATA points out that most of Chile is seismically or volcanically active, which has not prevented the country's numerous other hydroelectric projects from going ahead. “The Environmental Impact Study for Cuervo Hydroelectric Plant includes geological baseline studies with specific studies to establish the seismic and volcanological conditions and geological hazards in the project area,” says Christian Nuñez, environmental manager for Energía Austral, which runs the XSTRATA project. “To ensure the safety of the project, an engineering design was chosen that took into account the most extreme probable seismic scenario and the accelerations and landslides that could occur in this type of event. The Macá and Cay volcanoes, among others, have been incorporated into the risk evaluations,” Nuñez adds.

Ice floods

The threat is not only from below ground. In Patagonia, glacial lakes often overrun their banks and send meltwater surging downstream; such GLOFs are becoming more frequent with global warming, according to the country's climate scientists. There are currently five GLOFs in the North Patagonian ice field, with a large one spilling at the headwaters of the Baker River since 2008.



Activist. An opponent of dams, Peter Hartmann wants Patagonia to be a “reserve for life.”



Powering up. Companies plan to send electricity north to Santiago on more than 5000 towers.

“They are preparing to construct dams on what is probably the most unstable river system on the planet,” says Brian Reid, a limnologist at CIEP in the University of Austral, Valdivia. “To what extent it is active we don't know because it's never been studied.”

The GLOF analysis submitted in HidroAysén's EIA was “utter nonsense,” says Claudio Meier, a hydrological engineer at the University of Concepción. “They looked at water-level data for 1963 to 2007—less than 50 years—and extrapolated to obtain a 1-per-1000-year GLOF risk,” Meier says. “In fact, the peaks that they saw during the data period were just normal seasonal floods. The major GLOFs that occurred in that region were in 1962 and 2008; we've had six events there in the past 2 years.” The company needs to look at megaflood occurrences and come up with a risk for 1-in-5000-year and 1-in-10,000-year events, he says. GLOFs “have been much larger in the past than anything we're experiencing now” and have been known to flow at the extreme rate of 15,000 m³/s.

Reid also questions whether the dam could withstand the sediments a GLOF dumps, which both increase the flood levels

A Craving for Hydropower

The reason Chile must build at least seven new hydrodams, sponsors of the projects say, is that forecasts show that the country desperately needs more electricity—and that it must come from Chilean sources. Environmentalists and independent energy analysts have challenged this view; they argue that by improving efficiency and investing in renewable energy, Chile could find more than enough power within its borders for at least a decade—and without more dams. So far, however, energy planners have not been persuaded.

To meet future energy demands, according to April 2008 government projections, Chile needs to double its installed energy-generating capacity over the next decade and triple it by 2025. With virtually no coal and no oil or gas, the country imports more than 95% of its fossil fuels. This is one reason Chile has the most expensive energy on the continent and why leaders want to expand domestic energy production. Half the country's electricity comes from plants fueled by Argentinean oil or gas and Colombian coal. The other half comes from existing hydropower schemes, many of them in the central zone around the Biobío River.

In the summer of 2008–09, Argentina cut off gas supply to Chile at a time when months of drought had reduced Chile's hydropower capacity. The nation was plunged into blackouts. A powerful earthquake near Concepción in February again caused blackouts across the central grid after a single transmitter went down. Developing a new southern source of electricity became an attractive goal.

"The earthquake definitely boosted our chances of approval. ... We may have another year's delay, but we'll be approved," predicts an employee of HidroAysén, the company proposing to build five of the dams, speaking on condition of anonymity.

But a 2009 study by a consortium of Canadian and Chilean energy analysts disagrees. In their report, titled *Are Dams Necessary in Patagonia?*, Stephen Hall and his colleagues say that the country's projected electricity requirements to 2025 can be met by newly approved plans for coal plants. Even

without the plants, they argue, Chile could obtain the 3500 megawatts per year promised from new Patagonian dams through energy-efficiency measures (3041 MW, they calculate) and renewable energy development (4383 MW). The total gain would be twice that generated from the Patagonian dams, they say.

The green energy-conservation pitch has not persuaded Claudio Zaror, a chemical engineer at the University of Concepción and an energy adviser to the government. "Every year, the country needs an extra 500-MW capacity—another 8% a year," Zaror says. "We have to get that energy from somewhere. Environmentally and economically, hydropower is our only feasible option."

The situation is more perilous than some realize, Zaror says, because droughts are predicted to become more frequent and more severe across the central region, which includes the Biobío River, source of most of the nation's hydropower. Studies by the Global Change Research Center in Santiago completed in May 2009 predicted that average rainfall would decline in this region by 15% from the present by 2050 and that by 2065 the flow of the Maipo River would drop 70%—from an average of 170 m³/s to 60 m³/s. These conditions have already arrived, thanks to a severe drought that began in 2008

and from which Chile has yet to recover. Already, Zaror says, the drier climate caused by an El Niña ocean-current shift is affecting the region between Concepción and Santiago. "During the 2008 drought, less than 15% of the baseload was met by hydro, and we had to import diesel for the power plants at \$118 per barrel," Zaror says. He argues that Patagonia won't be affected as severely by drought: With 92% of the country's glaciers retreating, he predicts, the south will continue to enjoy strong river flow.

For Zaror, the issue is clear-cut. "Per capita income correlates closely with per capita energy consumption, so for a developing country, consumption will rise," Zaror says. "We have nearly 20% of the population living in extreme poverty at the moment. I want that number to decline, and we need energy for that."

—G.V.



Only one option. Government energy adviser Claudio Zaror believes Chile must embrace the hydrodams.

Icy flow. One of two glaciers in Patagonia that is not retreating, Perito Moreno supplies the Baker River with meltwater.



and reduce the hydrodam's life span by raising the reservoir bed and clogging the turbines. "A GLOF in Iceland in 1996 deposited 1% of the world's sediment load for a year in a 12-hour period, making it the second largest river in the world during that time. Would the HidroAysén dam cope with this? Could they open the gates in time?" Reid asks. He doesn't think there is a clear answer, noting that "I am the only person who's even studied suspended sediments during a GLOF."

HidroAysén says it is confident that its planned dam would withstand a GLOF, because it is designed to support a flow of 7000 m³/s. HidroAysén's experts—a range of in-house experts and contractors—calculate that a GLOF occurring during peak flow would double the rate to no more than 6000 m³/s, comfortably within the dam's margins.

Breaking the wilderness

One of scientists' big worries is that hydrodams and other development could mar unique, unstudied areas. For example, on the Baker River, one of the proposed dams "would flood large areas of peat bog based on volcanic ash soils and destroy a lot of unique wetland habitat that hasn't even been studied," Reid says. These areas are important habitats for fish and vulnerable to the practice of "hydropeaking," flooding and draining a reservoir. "Artificial water level fluctuations disturb the natural seasonal synchronicity in a lake," Meier says.

The effect of artificial daily pulses on fish can be devastating, says Evelyn Habit, a native fish biologist at the University of Concepción. "All the important spawning areas are in the wetland zone of daily flood and drop; if you lose spawning areas, you lose the species. Adults live in very deep layers



Vanishing. Large areas of unstudied ancient peat bog would be flooded by proposed dams.

and use the signal of water levels to know when to feed and reproduce, so if it's changing daily, it's unpredictable."

The wetland areas are also an important source of fish food—terrestrial debris—that would be lost if the forests are disconnected from the water by artificial floods and dams. "All our fish are benthos feeders, and they need large, woody debris. There's no information on these impacts in the EIA," Habit says. She's also concerned about how new roads and commercial development could affect unique fish species including, in the Yulton and Meullin lakes, the site of the proposed XSTRATA dam, a genetically distinct *Galaxias platei* species of primitive fish.

XSTRATA maintains that it has voluntarily set operating restrictions for its Cuervo plant to minimize the environmental damage from hydropeaking. "By maintaining variations within ranges that occur naturally in the existing banks, the company aims to limit the potential impact on the bank environment." Nuñez says that simulations indicate that the hydropeaking variations "will be achieved with similar minimum and maximum levels to the current lake system."

The Baker River is the most important river to protect, according to Habit, because of a historical quirk that has infused the system with uniquely rich biodiversity. During the last glacial period, 10,000 to 20,000 years ago, the river reversed direction, now flowing to the Pacific rather than the Atlantic. It contains "a unique population of fish that are endemic to Argentina," such as a diplomystes of a primitive catfish genus and *Odontesthes hatcheri* (silverside), a type of atheriniform, Habit says: "It seems crazy to me to make such a big alteration to such pristine ecosystems."

HidroAysén says it will replant trees elsewhere that are lost in its dam-building and "create an 11,560-hectare conservation area to safeguard the ecosystem and species." XSTRATA is also planning what it describes as "one of the most ambitious programs of reforestation with native species in Chile's history," to plant trees in parts of Patagonia that were burned by fires in the first half of the 20th century.

Nuñez, the company's environmental manager, also points out the greenhouse-gas benefits of hydro- versus fossil fuel-derived energy: "Energía Austral will have

it is to make a few private companies rich at the expense of our shared environment."

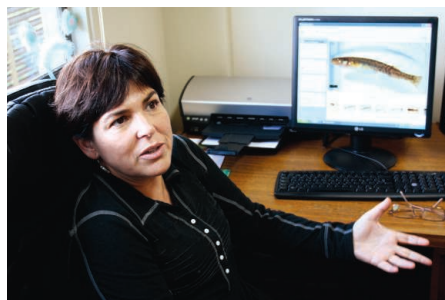
And it is perhaps this more than anything that lies at the heart of the standoff. Those against the megadam projects do not feel that the cause to which they are being asked to sacrifice their shared environment is worth it. Most of the energy generated, they claim, will be used for privately owned mining concessions, which include Barrick Gold's environmentally controversial large new mine at Pascua-Llama, and not for Chilean households.

Thousands took to the streets of Chile's cities last month, from Coyhaique to Santiago, protesting the dams. Their numbers were swelled by those opposed to recent public attempts by HidroAysén to pressure the government into fast-tracking approval for the projects. The company's projected costs are now pegged at \$7 billion. Thanks to another delay in the schedule requested by HidroAysén last month, the government will have until the end of the year to accept or reject the projects outright—or ask more questions.

Many believe that the decision ultimately rests with the conservative billionaire president, Sebastián Piñera, who may be as personally divided as his electorate. He is both a conservationist and a supporter of large private-enterprise projects like these. If the dams get the go-ahead, electricity production could begin as early as 2015; if not, Chile will be one of the few developing countries to choose to protect its natural environment over short-term financial gain.

—GAIA VINCE

Gaia Vince writes on environmental issues in the developing world at wanderinggaia.com.



Disruptive flow. Biologist Evelyn Habit fears that "hydropeaking" would devastate fish populations.

the capacity to displace from Chile's Central Interconnected System a total of 2.7 million tons of CO₂/year." HidroAysén has promised Patagonians an economic windfall if dams go forward: a reliable, cheap source of energy for the future.

Hartmann, standing under the three wind turbines that power the town of Coyhaique, says he's skeptical. "The same thing was promised for the people of Biobío before their dam was built; now they have the most expensive energy in the country," he says. "This project is not something to benefit the people of Chile;

PROFILE: NICHOLAS DODMAN

Can Dogs Behaving Badly Suggest A New Way to Treat OCD?

One of the world's most popular vets argues that research on animals with behavioral problems can offer insight into people with obsessive-compulsive disorder

NORTH GRAFTON, MASSACHUSETTS—

Walking from his office to the nearby clinic, Nicholas Dodman sports the traditional white coat of a physician. But his orange tie covered with dogs is a hint that the patients he'll see later today aren't of the two-legged variety. Indeed, the 64-year-old veterinarian and founder of the Tufts University Animal Behavior Clinic is soon visiting with Baron, an 8-year-old brown Doberman pinscher who began compulsively licking himself after a sarcoma led to amputation of a hind leg.

Dobermans are an "oral breed, very lick, sucky, chewy," Dodman says, and some will lick their flanks or limbs until raw and bleeding. Although the vet concludes that Baron isn't dangerously compulsive, he recommends, in addition to exercise and "environmental enrichment," that the animal begin taking Prozac and memantine, a drug approved for the treatment of Alzheimer's disease symptoms in people. Next up for Dodman is Theo, a Bernese mountain dog who has suddenly become fearful around strangers; then Ginger, an overly aggressive Australian shepherd taking Prozac to help her behave; and Quincy, an anxious golden retriever who whimpers when his owner leaves the room. It's all in a day's work for the author of best-sellers such as *Dogs Behaving Badly*, *The Dog Who Loved Too Much*, and *The Cat Who Cried for Help*.

A fast, fluent talker with the charm of a good storyteller, Dodman regularly conducts seminars on pet behaviors for vets, animal trainers, and pet owners. He writes magazine columns; blogs for a dog Web site; runs another Web site on pets with his wife, also a veterinarian; and is a spokesperson for a spray that eliminates cat-litter odors. As such, it is perhaps easy to dismiss Dodman as the Oprah of the pet world.

But the veterinarian balances this celebrity with innovative animal research that appears in scientific journals. Earlier this year, for example, he and colleagues reported in *Molecu-*

lar Psychiatry that variations in the gene for a protein involved in central nervous system development may underlie "canine compulsive disorders" in Doberman pinschers.

Moreover, Dodman contends that animal compulsions bear relevance to human obsessive-compulsive disorders and that memantine may help people with OCD. A small clinical trial just completed supports his view, suggesting that the compound could be the first new OCD treatment in decades. Dodman even argues that studying aberrant animal behaviors may suggest



Puppy love. Nicholas Dodman's passion for animals and perspective on their misbehavior have made him famous.

treatments for a wide range of psychiatric conditions. Yet it hasn't been easy to convince other scientists. "Trying to get this concept over is like rolling a rock up a hill," he laments.

Some researchers are now at least paying attention. Colleagues in nearby Boston have begun brain-imaging studies of his compulsive Dobermans. And Dennis Murphy, chief of clinical science at the National Institute of

Mental Health (NIMH) in Bethesda, Maryland, is examining the Doberman-highlighted gene in people with OCD. The dog study "gives us a specific target to look at for compulsive behaviors in humans," Murphy says. "This finding is too important to ignore."

The eureka moment

Dodman grew up in England, and an affinity for animals led him to train to become a vet. "I felt really strongly about animals being sentient beings and abhorred animal abuse. It just felt right at the time ... to spend my days trying to help them as best I could," he says.

Dodman's initial veterinary focus was surgery and anesthesiology. But after moving to the United States in 1981 to join the Tufts veterinary school, he struck up a friendship with Tufts pharmacology researcher Louis Shuster. Together, they became interested in developing treatments for horses exhibiting so-called stereotypic behaviors: repetitive, seemingly pointless actions such as pacing, cribbing, and wood-chewing. They hypothesized that the physical activity of these "stall vices" increased production of pleasurable endorphins, making the behaviors a form of equine self-medication. Dodman and Shuster found proof for their theory: Giving horses opioid antagonists, which block endorphin action, stopped the behaviors.

It was a "eureka moment," says Dodman. "That one experiment is what turned me into a behaviorist." It also led to a patent on treating equine stereotypic behavior with an opioid antagonist, the first of more than a dozen patents that Dodman, usually in concert with Shuster, has generated for Tufts.

Shuster and Dodman quickly began investigating treating stereotypic behaviors in other animals. Bull terriers often exhibit unnaturally obsessive "predatory" behavior, for example. They'll chase their tails or objects such as balls for long periods, compulsively carry and chew objects, and eat inedible material. Some cats hoard or compulsively lick their paws. Even birds may chronically pick their feathers, which, says Dodman, resembles trichotillomania, a condition in which people obsessively play with, and pull out, their hair.

Shuster and Dodman learned that besides blocking receptors for endorphins, opioid antagonists also block NMDA receptors, which mediate responses to the neurotransmitter glutamate. So they tried other glutamate receptor blockers, such as dextromethorphan—the cough medicine—and memantine, on compulsive behaviors in other animals. They found that combining memantine with fluoxetine, better known as the antidepressant Prozac, was effective

in halting compulsive scratching behavior induced in mice. The Tufts team then tried memantine with or without fluoxetine on 11 dogs with various compulsive manifestations: tail-chasing, circling, snapping at invisible flies, and light/shadow chasing. Seven of the dogs improved.

About 7 years ago, Dodman and Shuster began talking with psychiatrist Michael Jenike, head of the Obsessive Compulsive Disorders Institute at McLean Hospital in Belmont, Massachusetts. OCD researchers had long focused on the neurotransmitter serotonin. Increasing serotonin levels with fluoxetine, a so-called selective serotonin reuptake inhibitor, can sometimes reduce symptoms of the disorder. But a subtle shift in thinking had begun developing among OCD investigators. In addition to the memantine animal work, studies of people had implicated glutamate signaling in OCD. A Yale University team, for example, had begun to test a drug that reduces glutamate release, riluzole, in people with OCD.

Given the lack of new treatments for OCD, Jenike tried memantine on a few of his patients and was impressed. He, Dodman, Shuster, and colleagues then launched a case-control study, comparing 22 people with OCD receiving standard treatment at the clinic with 22 similarly treated OCD patients who were also given memantine. The memantine takers, but not the controls, had a significant decrease in their OCD symptoms, the researchers reported in the February *Journal of Clinical Psychopharmacology*.

Jenike has now raised money to conduct a larger placebo-controlled trial of memantine. Despite all that, he remains skeptical that animal compulsions are directly analogous to OCD. "I have a hard time with the concept of a dog biting on his leg and calling it OCD. With OCD, you need to know what's going on in the head. It's kind of a big leap for me," he says.

Spontaneity is good

Researchers have traditionally sought to create animal models of mental health problems by, for example, genetically engineering or stressing rodents. Yet such models are viewed with caution. "Trying to create an animal model defined by behaviors which resemble human psychopathology is a tough one. ... They capture, at best, only a fraction of the human syndrome," notes Thomas Insel, director of NIMH.

Dodman contends that spontaneously occurring behavioral problems in animals offer a much more promising avenue of study than inducing a disorder does. "If I look at the *DSM* [psychiatry's *Diagnostic*



Unstoppable. Animals often exhibit compulsive behavior such as chewing wood, licking themselves, and feather-pulling.



and Statistical Manual], there's hardly a disorder in there that I don't see in animals," he says. (Schizophrenia, he later acknowledges, is one of the exceptions.)

Dodman argues that there's a common theme to human OCD and compulsive animal behavior: instincts gone awry. "Human OCDs relate to hunting and gathering," he asserts. Hoarding behavior, for example, is gathering run amok. Animal compulsions are similarly keyed to their basic instincts, according to Dodman. Grooming can go overboard, resulting in aberrant paw licking in cats and dogs and feather-pulling in birds.

Convinced that there's also a shared genetic component among people and animals for compulsive behavior, Dodman has been laying the foundation to confirm that theory for more than decade. In 2000, Dodman joined forces with geneticist Edward Ginns of the University of Massachusetts Medical School in Worcester, who was interested in screening select populations of people, such as the Amish, and animals, such as specific dog breeds and purebred horses, for disease genes. They collected hundreds of DNA samples from horses and dogs with severe compulsions and waited for technology to ripen. By 2007, the canine genome had been sequenced, and Dodman and Ginns, with the help of the Broad Institute in Cambridge, Massachusetts, began to probe the DNA samples of Dobermans with obsessive sucking or licking behavior. The search paid off at the end of last year when the team found that a variant of the gene for a protein called cadherin-2 (or neural cadherin) was over-represented in extremely compulsive Dobermans compared with normal ones.

Studies have suggested that cadherin-2 assists brain development, apparently helping neurons form synaptic connections. And Dodman believes the memantine and cadherin gene stories converge to further implicate glutamate in compulsive behaviors: Cadherins contribute to the formation and action of NMDA receptors, the target of memantine. Compulsive behavior "isn't all about the NMDA receptor, [but] it certainly isn't all about serotonin," currently the prime target for OCD drugs, he says.

Dodman is now anxiously awaiting news from various collaborators. NIMH's Murphy should have results soon on whether the human version of the *cadherin-2* gene or related cadherin genes are implicated in people with OCD. And Elaine Ostrander at the National Human Genome Research Institute in Bethesda is almost done with a genomewide survey of bull terriers who are severe tail chasers.

Dodman is particularly excited about the bull terrier study. Some members of this breed not only exhibit compulsive stereotypic movements but also are asocial and withdrawn, prone to seizures and "trances," and display episodes of explosive aggression, all traits seen in autistic people. Dodman notes that a recent genetic study of people with autism has also implicated cadherins. "Things are getting curiouser and curiouser," he says. That rock may be about to inch farther up the hill.

—CONSTANCE HOLDEN AND JOHN TRAVIS

Constance Holden, a 40-year veteran of *Science's* News department, was killed in a traffic accident during preparation of this story, which was completed by John Travis. She is remembered by her colleagues and scientists at www.sciencemag.org/extra/holden/

LETTERS

edited by Jennifer Sills

Bracing for Oil

UNEASINESS HAS SETTLED ON THE UNIVERSITY OF MIAMI'S ROSENSTIEL School of Marine and Atmospheric Science campus as we watch the oil spill slick creep across the Gulf. Although we are on Florida's eastern side, the currents will likely carry the oil to our shores. What would become of the beach surrounding our campus, and the reefs just offshore that we measure and map and monitor regularly?

On campus, we have set up our own drilling rigs, drilling not for



Creeping closer. Weeks after the April 2010 Gulf of Mexico oil spill began, a wave of oily tarballs washes over a footprint in Orange Beach, Alabama.

oil, but for clean seawater. The tanks housing our corals and fish draw water directly from Biscayne Bay, and we hoped that by digging wells, we could use sand to filter out the incoming pollutants. Results are mixed: One of the wells has produced water of high quality, but the other has high loads of inorganic nutrients, rendering its utility questionable and the security of the water supply uncertain.

It is hard to predict how the oil will affect our reefs. Many of the studies on the effects of oil and dispersants on corals were done in the 1970s and early 1980s (1, 2). Although many sublethal effects were reported, acute mortality in connection with oil alone was low. The mucous layer of corals may allow them to slough off oil before it causes damage. The dispersants are more toxic; they can dissolve the corals' mucous layer, which would allow chemicals to penetrate the tissues. Our predictive capacity is further compromised by the wide range of secondary stressors, including coral bleaching, ocean acidification, disease outbreaks, and algal overgrowth. These factors might make the corals even more vulnerable, particularly to toxic exposures.

Powerless, we wait and watch, trying to enjoy our beach but always mindful of the days, perhaps not far off, when tarballs will mingle with the seaweed washed ashore. As we work to mitigate this disaster, we must go beyond a clean up and demand better protection for these devastated and dwindling ecosystems.

RACHEL N. SILVERSTEIN

Rosenstiel School of Marine and Atmospheric Science, University of Miami, Miami, FL 33149, USA. E-mail: rsilverstein@rsmas.miami.edu

References

1. Y. Loya *et al.*, *Oil Petroch. Poll.* **1**, 157 (1983).
2. Y. Loya, B. Rinkevich, *Mar. Ecol. Prog. Ser.* **3**, 167 (1980).

Evolution of Fairness: Cultural Variability

J. HENRICH *ET AL.* ("MARKETS, RELIGION, Community size, and the evolution of fairness and punishment," Research Articles, 19 March, p. 1480) have shown that market integration and participation in world religion covary with fairness. The authors suggest that their results support cultural evolution theories and contradict the hypothesis that successful social interactions in large-scale societies arise to a large extent from an evolved psychology. We believe that their conclusion is based on too simple a view of human morality.

Much research in behavioral economics supports the idea that humans have a

sense of fairness that aims to equilibrate exchanges among individuals. In economic games where money needs to be distributed, for instance, people carefully respect everyone's rights over the stake: If the common good is produced by a single person, she is granted more rights over the money (1, 2); similarly, the most productive partner during the joint production phase is favored (3, 4).

Economic games are notoriously underdetermined: Participants are given a lump of money to distribute with no information as to where it comes from, who owned it in the first place, who the receiver is, and so on. As the authors have noted in previous papers (5), participants have no choice

but to fill this informational gap by drawing on their everyday life experience. Because participants in more market-integrated societies have more experience in sharing goods and investing with others, they spontaneously attribute more rights to the other participant and consequently allow her more money (6).

This explanation fits better with the economic literature on institutions and cooperation. Contrary to what the authors suggest, Nobel Prize-winning economists Douglas North (7) and Elinor Ostrom (8) have shown that cultural variability in cooperation is not explained by different norms but rather by different systems of incentives (reward and penalties) organized by local communities or

States. Thus, an innate preference for fairness is fully compatible with Henrich *et al.*'s results. It is also theoretically more parsimonious and supported by more empirical evidence.

NICOLAS BAUMARD,^{1*} PASCAL BOYER,²
DAN SPERBER³

¹Department of Anthropology, Institute of Cognitive and Evolutionary Anthropology, University of Oxford, Oxford OX2 6PN, UK. ²Department of Psychology, Washington University, St. Louis, MO 63130, USA. ³Department of Philosophy, Central European University, 1051 Budapest, Hungary.

*To whom correspondence should be addressed. E-mail: nbaumard@gmail.com

References

1. R. J. Oxboby, J. Spraggon, *J. Econ. Behav. Org.* **65**, 703 (2008).
2. T. L. Cherry, P. Frykblom, J. F. Shogren, *Am. Econ. Rev.* **92**, 1218 (2002).
3. N. Frohlich, J. Oppenheimer, A. Kurki, *Public Choice* **119**, 91 (2004).
4. A. W. Cappelen, A. D. Hole, E. O. Sorensen, B. Tungodden, *Am. Econ. Rev.* **97**, 818 (2007).
5. J. Henrich *et al.*, *Behav. Brain Sci.* **28**, 795 (2005).
6. N. Baumard, D. Sperber, *Behav. Brain Sci.* **33**, 60 (2010).
7. D. C. North, *Institutions, Institutional Change, and Economic Performance* (Cambridge Univ. Press, Cambridge, 1990).
8. E. Ostrom, *Governing the Commons: The Evolution of Institutions for Collective Action* (Cambridge Univ. Press, Cambridge, 1990).

Evolution of Fairness: Rereading the Data

J. HENRICH *ET AL.*'S RESEARCH ARTICLE ("Markets, religion, community size, and the evolution of fairness and punishment," 19 March, p. 1480) is a valuable addition to the growing literature testing behavioral hypotheses through careful cross-cultural measurement. However, the data they report falsify their theory. The authors propose that increases in third-party punishment of unfairness drove an increase in fairness norms, enabling the emergence of large-scale market economies. Critical to this theory is their hypothesis that exposure to third-party punishment actually elicits an increase in fairness. Relevant to evaluating this hypothesis, the authors conducted two near-parallel economic games: a Dictator Game (in which a "dictator" unilaterally divides a wind-fall gain with another person—a measure of fairness) and a Third-Party Punishment Game (the Dictator Game with the addition of exposure to possible third-party punish-

ment). Their central hypothesis requires that adding punishment to the Dictator Game will increase fairness, but their data show that the addition of punishment decreases fairness (p. 1483). This finding unambiguously refutes their central hypothesis.

Henrich *et al.* also assert that their data—showing patterned cultural variability in cooperation—can determine whether modern levels of generosity and altruism are driven by an evolved social psychology or by cultural processes. The authors claim that their data decisively favor the cultural processes hypothesis. Yet nothing in their data can test (even in principle) whether it is psychological or cultural processes (or both) that cause these cross-cultural differences. Only long-abandoned instinct-as-reflex theories expect invariant responses in the face of different social inputs. By contrast, modern adaptationist theories predict that our evolved social psychology will be calibrated by relevant environmental inputs. Many of these inputs—such as the local value of long-term cooperative relationships and the fidelity of reputations—are likely to covary with market integration, making it at least as likely that an evolved, context-sensitive social psychology is driving the results that the authors observe. That is, psychological and cultural theories both predict cross-cultural variation.

ANDREW W. DELTON, MAX M. KRASNOW,
LEDA COSMIDES, JOHN TOOBY*

Center for Evolutionary Psychology, University of California, Santa Barbara, CA 93106–9660, USA.

*To whom correspondence should be addressed. E-mail: toooby@anth.ucsb.edu

Response

OUR RESEARCH ARTICLE DOES NOT ARGUE that elements of an evolved social psychology are unimportant, as Baumard *et al.* suggest. Nor do we believe, as Delton *et al.* propose, that purely genetically evolved mechanisms, rooted in kinship and reciprocity, are sufficient to account for the massive expansion of cooperation in the past 10 millennia and the diversity of human sociality. We argue that understanding this expansion requires the integration of work on both cultural and genetic evolution.

Baumard *et al.* misunderstand the work we cite on the evolution of norms (1–4), as well as that of North and Ostrom. The cited work asks two questions of innate learning heuristics: (i) Under what conditions can these adaptive learning mechanisms yield self-reinforcing stable equilibria ("norms") at which individually costly behaviors are sustained, and (ii) what happens when groups stuck at different stable equilibria interact and compete (5)? Thus, we draw on work that explicitly theorizes the origins and evolution of "incentive systems" given what we know empirically about human psychology and learning.

Both our theoretical approach and our empirical evidence are convergent with approaches suggested by North and Ostrom, who have emphasized the importance of norms, institutions, and learning and culture [pp. 89, 17, and 75 in (6); p. 75 in (7); pp. 42, 86, 87, and 135 in (8)].

Baumard *et al.* claim that much research supports the idea "that humans have a sense of fairness," but the four papers they cite are limited to Dictator Games among undergraduates. Although we strongly suspect that there are innate elements to human fairness (9, 10), the empirical findings cited are neither applicable to our recent paper nor established beyond Western undergraduates, who are but one population in a broad spectrum of human diversity (11, 12).

Baumard *et al.*'s concerns about underdetermination in our economic experiments are unfounded; in the protocols we administered across 15 diverse populations, property rights (money was "given to the pair"), the origins of the money, and what players know (and do not know) about the receiver were made explicit and held constant across sites. Analyses using our measures of comprehension and formal education (10), as well as our post-game interviews, revealed no hint that systematic misunderstandings of the instructions (e.g., regarding property rights) affected the findings. Of course, if different populations hold different learned and internalized norms from daily life regarding property rights, this would support our theoretical view.

Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the previous 3 months or issues of general interest. They can be submitted through the Web (www.submit2science.org) or by regular mail (1200 New York Ave., NW, Washington, DC 20005, USA). Letters are not acknowledged upon receipt, nor are authors generally consulted before publication. Whether published in full or in part, letters are subject to editing for clarity and space.

Finally, Baumard *et al.* explain the strong statistical relationship we found between markets, religion, and community size by noting that people in market societies have more experience with “sharing.” Empirically, this view is inconsistent with substantial anthropological evidence for intensive food sharing, especially in the smallest-scale societies (13). Delton *et al.* also refer to a relationship between market integration with “cooperative relationships” and the “fidelity of reputations,” implying that in the smallest-scale human societies (such as foragers), long-term cooperative relationships have relatively less value than among Americans, and that reputations in these face-to-face communities have lower fidelity than among Western urbanites. Such a proposal runs contrary to all available evidence (14, 15).

Delton *et al.*'s concerns about the relationship between our Dictator Game (DG) and Third-Party Punishment Game (TPG) stem from their belief that our approach assumes that only social norms affect experimental results. However, we argue that culturally evolved norms influence decision-making along with other factors (such as material costs and benefits), which is why we equalized the stakes across sites and controlled for income and wealth. Reflecting this, we proposed that the drop in TPG offers relative to the DG arises from the interaction of two factors (pp. 56 to 61 in our supporting online material). First, empirical work suggests that when punishments or rewards are

added to a situation, they may reduce intrinsic motivations toward some goal or norm (16). If punishing is structurally limited or weak, the net effect of adding this threat to a social interaction may be to reduce motivations to be social. However, if the punishment threat is increased, the net result will be greater social behavior than in the nonincentivized situation. Second, our TPG adds only limited punishment to a DG. The most our punishers can do is to reduce a players' take by 30%. Consequently, it seems likely that offers went down in our TPG because the game adds a punishment threat (reducing intrinsic motivations) but provides limited punishment opportunities—creating insufficient compensatory incentives. This explains why punishment increased offers slightly in the Ultimatum Game (UG), relative to the DG, whereas it reduced offers in the TPG; the UG has potent punishment. Thus, TPG mean offers could be raised by increasing the opportunities for punishment. This underlines the fact that our experiments were not designed to assess the relative efficacy of third-party punishment on prosocial outcomes, but rather to compare a fixed punishment opportunity across populations. Analyses of the differences in DG and TPG offers (table S18 in our supporting online material) suggest that adding limited punishment reduces those prosocial motivations linked to world religions.

We agree that applying evolutionary theory can generate context-dependent theories

about psychology (9, 17). However, when one takes the classic models seriously, without applying ad hoc verbal extensions, the implications are clear: Genetic mechanisms based on kinship, reciprocity, and signaling are alone insufficient to account for the scale, temporal trajectory, and variation in human cooperation (9, 18, 19).

JOSEPH HENRICH,^{1*} JEAN ENSMINGER,² RICHARD MCELREATH,³ ABIGAIL BARR,⁴ CLARK BARRETT,⁵ ALEXANDER BOLYANATZ,⁶ JUAN CAMILO CARDENAS,⁷ MICHAEL GURVEN,⁸ EDWINS GWAKO,⁹ NATALIE HENRICH,¹⁰ CAROLYN LESOROGOL,¹¹ FRANK MARLOWE,¹² DAVID TRACER,¹³ JOHN ZIKER¹⁴

¹Departments of Psychology and Economics, University of British Columbia, 2136 West Mall, Vancouver, BC V6T 1Z4, Canada. ²Division of the Humanities and Social Sciences, California Institute of Technology, Pasadena, CA 91125, USA. ³Department of Anthropology, University of California, Davis, CA 95616, USA. ⁴Centre for the Study of African Economies, Department of Economics, University of Oxford, Manor Road, Oxford OX1 3UQ, UK. ⁵Department of Anthropology, University of California, 341 Haines Hall, Box 951553, Los Angeles, CA 90095, USA. ⁶Department of Anthropology, College of DuPage, Glen Ellyn, IL 60137, USA. ⁷Facultad de Economía, Universidad de Los Andes, K1 Number 18A-70, Bogotá, Colombia. ⁸Department of Anthropology, University of California, Santa Barbara, CA 93106, USA. ⁹Department of Sociology and Anthropology, Guilford College, 5800 West Friendly Avenue, Greensboro, NC 27410, USA. ¹⁰Centre for Health Evaluation and Outcome Sciences, Providence Health Care Research Institute, Vancouver, BC V6Z 1Y6, Canada. ¹¹George Warren Brown School of Social Work, Washington University, 1 Brookings Drive, St. Louis, MO 63130, USA. ¹²Department of Anthropology, Florida State University, Tallahassee, FL 32306, USA. ¹³Department of Anthropology, University of Colorado, Post Office Box 173364, Campus Box 103, Denver, CO 80217, USA. ¹⁴Department of Anthropology, Boise State University, 1910 University Drive, Boise, ID 83725, USA.

CORRECTIONS AND CLARIFICATIONS

This Week in Science: “Glacial gas” (25 June, p. 1609). The first five words should not have run. The first sentence should read, “A series of sudden and large warming episodes, called Dansgaard-Oeschger events, interrupted the cold conditions of the last glacial period.” The sentence has been corrected in the HTML version online.

Editors' Choice: “Caught in the net” by C. Ash (11 June, p. 1328). In the second sentence, “apatite skeletons” should have been “aragonite skeletons.”

News of the Week: “APA seeks to overhaul personality disorder diagnoses” by C. Holden (12 March, p. 1314). Andrew Skodol was incorrectly identified. He is a psychiatrist at the University of Arizona College of Medicine in Tucson.

Cover Caption: (26 February, p. 1049). The cover caption did not sufficiently acknowledge the airborne laser mapping project called “B4.” In the image credit, the following should have been added parenthetically after “the B4 Project”: “(Ohio State University, U.S. Geological Survey, National Center for Airborne Laser Mapping, UNAVCO, and Optech International; www.earthsciences.osu.edu/b4).”

Reports: “Slip in the 1857 and earlier large earthquakes along the Carrizo Plain, San Andreas Fault” by O. Zielke *et al.* (26 February, p. 1119). In note 11, the description of the source of the data used in this study was incomplete, and in note 28, the airborne laser mapping project called “B4” was insufficiently acknowledged. Note 11 should have read: “This LiDAR point cloud was gathered by the National Center for Airborne Laser Mapping using an Optech ALTM 3100 in a Cessna 310 aircraft flying at 600 m above ground level with a 70-kHz pulse rate and $\pm 20^\circ$ scan angle. The ~ 1 -km-wide survey comprised five overlapping swaths. These data are version 1.01 of the B4 project data, which use Kendrick's 26 November 2005 GPS processing with the KARS software. Points with location accuracies of better than a few decimeters have densities of 2 to 4 per m^2 along the 1857 reach, which allowed us to download 0.25 to 0.5 m per pixel Digital Elevation Models computed using a local binning approach (www.opentopography.org).” The following line should have appeared at the end of note 28: “The ‘B4 Project’ (www.earthsciences.osu.edu/b4) collected LiDAR point cloud data along the southern San Andreas and San Jacinto Faults. Data acquisition and processing were performed by the National Center for Airborne Laser Mapping (NCALM). The project was led by Ohio State University and USGS with funding from the Division of Earth Sciences Geophysics program at the NSF. Optech International contributed the ALTM3100 laser scanner. UNAVCO and Southern California Integrated GPS Network assisted in GPS ground control. Numerous volunteers and landowners made the project possible.”

References

1. J. Henrich, R. Boyd, *J. Theor. Biol.* **208**, 79 (2001).
2. K. Panchanathan, R. Boyd, *Nature* **432**, 499 (2004).
3. H. Gintis, E. A. Smith, S. Bowles, *J. Theor. Biol.* **213**, 103 (2001).
4. J. Henrich, *J. Econ. Behav. Organ.* **53**, 3 (2004).
5. J. Henrich, *Science* **312**, 60 (2006).
6. D. C. North, *Structure and Change in Economic History* (Norton, New York, ed. 1, 1981).
7. D. C. North, *Understanding the Process of Economic Change* (Princeton Univ. Press, Princeton, NJ, 2005).
8. D. C. North, *Institutions, Institutional Change, and Economic Performance* (Cambridge Univ. Press, New York, 1990).
9. N. Henrich, J. Henrich, *Why Humans Cooperate: A Cultural and Evolutionary Explanation* (Oxford Univ. Press, Oxford, 2007).
10. J. Henrich *et al.*, *Science* **312**, 1767 (2006).
11. J. Henrich, S. J. Heine, A. Norenzayan, *Behav. Brain Sci.*, 10.1017/S0140525X0999152X (2010).
12. B. Herrmann, C. Thöni, S. Gächter, *Science* **319**, 1362 (2008).
13. M. Gurven, *Behav. Brain Sci.* **27**, 543 (2004).
14. J. Henrich *et al.*, *Behav. Brain Sci.* **28**, 838 (2005).
15. F. Marlowe, *The Hadza: Hunter-Gatherers of Tanzania: Origins of Human Behavior and Culture* (Univ. of California Press, Berkeley, CA, 2010).
16. S. Bowles, *Science* **320**, 1605 (2008).
17. M. Gurven, *Behav. Brain Sci.* **27**, 572 (2004).
18. R. Boyd, P. J. Richerson, *Philos. Trans. R. Soc. Ser. B* **364**, 3281 (2009).
19. E. Fehr, J. Henrich, in *Genetic and Cultural Evolution of Cooperation*, P. Hammerstein, Ed. (MIT Press, Cambridge, MA, 2003), pp. 55–82.

PLANT SCIENCE

A Plant Breeder's History of the World

Alan B. Bennett

“‘Hybrid’ is derived from the Latin *hybrida*, meaning a cross between a tame sow and a wild boar, a word

possibly related to the Greek *hubris*, meaning pride but also an outrage against nature.”

In *Hybrid*, Noel Kingsbury builds on this theme by exploring the conceptual breakthroughs that led people to accept that they could and should improve plants for human uses as well as the sci-

entific breakthroughs that progressively made crop improvement possible and, ultimately, powerful. Artfully linking human cultural evolution and the 10,000-year history of plant breeding, Kingsbury (an English horticulturalist and writer) moves fluidly between the art and science of plant breeding and the growth of increasingly complex human society and politics. He convinced me, at least temporarily, that plant technologies have been a major contributor to many important developments in human history. For example, in his description of the disastrous “days of madness” when Trofim Lysenko dominated plant breeding in the Soviet Union, he notes “[i]t has even been suggested that Lysenko’s influence so damaged Soviet agriculture that it, more than any other single factor, led to the demise of Communism.” Although many of Kingsbury’s ideas linking the stories of plant breeding and human civilization could undoubtedly be challenged, they create provocative food for thought and expand the book’s context beyond that of a technical history.

The history of plant breeding starts at the dawn of agriculture, when a few crops, in far-flung parts of the world, appear to have been almost simultaneously domesticated. Early farmers recognized distinctive new varieties that arose from sudden mutations or novel hybrids. Then, either consciously or unconsciously, farmers preferentially propagated the most favorable new types. Following the domestication of several major crops, plant improvement was painfully slow. Kingsbury attributes this general lack of progress to a variety of reasons. People may have remained unaware that humans could make

changes in living organisms, and the knowledge of how to make desired changes certainly did not exist. Societies facing rising

demands for crops found it harder to expand production than to conquer new lands or to encourage commerce; thus political investments favored the military and trade rather than agricultural improvement. For several millennia, the returns from improving agricultural crops were far too small and too slow to be widely appreciated by rapidly developing societies.

Much changed during the 17th and 18th centuries. In Europe during the Age of Enlightenment, researchers sought to understand nature and to manage it for human benefit. Experimentation and the concept that humans could select for new and useful plants were coupled with population growth and urbanization that provided a powerful motivation to expand crop productivity. Although this era of plant improvement preceded any knowledge of genetics, botanical science was emerging as a powerful partner to agriculture. Experimental approaches to creating hybrids identified the physical mechanisms of pollination, began to define the limits of hybridization between closely related species, and noted the phenomenon of hybrid vigor. The era witnessed the birth of systematic plant breeding.

Although there is a vast and interesting history of crop cultivation before the dawn of the 20th century, there is little question that the work of Darwin and Mendel created the scientific foundation for plant breeding that led to its explosive impact over the past 100 to 150 years. As Kingsbury notes, Mendel provided a framework for predictability in selective breeding and, perhaps most important, illustrated the role and importance of statistics in designing and evaluating breeding experiments. Kingsbury devotes several chapters to the fascinating history of 20th-century efforts to improve the productivity, reliability, and nutrition of crops. Among other topics, he discusses the rise of corn and of first-generation hybrids, mutation breeding, wide-species crosses and genetic introgres-

sion, the Green Revolution, and the challenges of genetic resource preservation and intellectual property rights that now play major roles in the plant breeding enterprise.

Plant breeding has a long history that is peppered with controversies and with the often interesting and powerful personalities who were involved in these disputes. For example, George Beadle and Paul Mangelsdorf, two of the most prominent plant geneticists of the 1930s to 1950s, argued over the domestication of corn. Their decades-long debate was finally settled in Beadle’s favor after his retirement (when he had the time to evaluate 50,000 maize-teosinte hybrids over two generations). Kingsbury weaves personal sketches of many other individuals into his history. Some are well known, such as William Bateson, the principal advocate for Mendelism in Britain; Liberty Hyde Bailey, who developed agriculture as an applied science at Cornell University; Luther Burbank, the intuitive breeder and showman; and John Chapman, “Johnny Appleseed,” supplier of cider

Hybrid

The History and Science of Plant Breeding

by Noel Kingsbury

University of Chicago Press, Chicago, 2009. 509 pp. \$35, £24. ISBN 9780226437040.



Golden grain. Field of hybrid corn, Iowa.

apples. Readers will become more familiar with other, less-known names: e.g., the family dynasties of the Vilmorins (France; especially important for cereals) and Wallaces (Iowa; corn), Antoine Duchesne (the 18th-century French botanist who worked with strawberries), and Rowland Biffen (the British botanist who first applied Mendelian genetics to improve crops, in particular wheat).

It is interesting that Bailey is also credited with inventing the term “plant breeding” and training students through a combination of “practical fieldwork and research based on sound scientific principles”—and this was just over 100 years ago. Both the discipline and the profession were major growth industries for the first three-quarters of the past century, but there are warning signs that they now face stresses from declining public investment and graduate training oppor-

The reviewer is at the Department of Plant Sciences and Public Intellectual Property Resource for Agriculture, University of California, Davis, CA 95616, USA. E-mail: abbenett@ucdavis.edu

tunities. Much of the disciplinary interest in plant breeding has migrated to the molecular biology of plants and of the professional interest, to agricultural biotechnology. Kingsbury stops his narrative short of the contributions from genetic engineering. He does not, however, ignore genetic engineering of crops but uses the history of plant breeding to place current genetic technologies in a broader context. *Hybrid* provides an informative tour of plant breeding through time, its interface with society and cultural evolution, and the people who contributed to this history. Kingsbury's account should be required reading for students preparing for a future as a plant breeder, geneticist, or molecular biologist. Fortunately, that requirement should prove unnecessary—the book is engaging at many levels, and I expect many scientists and lay readers to pick it up on their own accord.

10.1126/science.1192333

ANTHROPOLOGY

Cultivating Chaos

Saleem H. Ali

Searching for unifying causality to current environmental and social problems is a compelling endeavor for scientists who are intrepid enough to become public intellectuals. Tracing our successes and failures back to some momentous historical episodes has been attempted in various forms. In *Collapse*, Jared Diamond used case analyses of numerous societies to posit a grand theory of environmental determinism (1). Earlier works along similar lines include archaeologist Joseph Tainter's *The Collapse of Complex Societies* (2) and historian Clive Ponting's *A Green History of the World* (3).

It is within this genre of panoramic narrative that Spencer Wells seeks to plant *Pandora's Seed*. A population geneticist, he starts the book with a fundamental question: "Is there some sort of fatal mismatch between Western culture and our biology that is making us ill?" Empirically testing such a question is impossible, but through a convergence of narratives

Pandora's Seed
The Unforeseen Cost
of Civilization

by Spencer Wells

Random House, New York,
2010. 251 pp. \$26, C\$31.
ISBN 9781400062157.
Allen Lane, London. £20.
ISBN 9780713997552.

from disciplines as far afield as anthropology and psychiatry, Wells traces back our current societal predicament to the Neolithic advent of agriculture around 10,000 years ago. Using the allure of Greek mythological imagery, he suggests rather astonishingly that all sorts of human failings from obesity to terrorism can be traced back to a move away from hunter-gatherer lifestyles when humans decided to farm rather than forage.

In the age of *Avatar*, this storyline is indeed likely to resonate with the public, but it will raise many eyebrows among scientists across a wide spectrum. The romantic vision of hunter-gatherer lifestyles Wells presents misses many empirical findings. For example, the assumption that early humans were focused on need-based lifestyles and not interested in luxuries has been challenged

in various ways. Some of the earliest mining in the world, traced back to around 40,000 years ago in Swaziland's Lion Cave, was for ornamental ochre rather than for any particular biological need. Aggrandizing behavior has also been observed in hunter-gatherer societies, recently exemplified by the discovery of gold artifacts among Andean hunter-gatherer remains (4).

The importance of intergroup conflict at early stages in our history is another contentious topic. Although Wells concedes that hunters and gatherers did engage in battles, he minimizes this matter. Where the empirical evidence points toward mortality from trauma, he suggests that this was due to "accidental falls and drownings." Where he is lamenting the stresses of modern life, he dismisses the stress of hunting as "short-term" and actually positive because it produces adrenaline, which arguably boosts immune response. Yet such potentially salubrious adrenaline rushes could just as well be produced with video games or watching the stock market. Frequent skirting of confounding factors undermines Wells's narrative, which is otherwise very rich in descriptive detail and valuable charts.

It is worth considering one of the overarching concepts provided by the book, that of "transgenerational power." Wells suggests that human abilities are now so powerful that the activities of those currently alive



Seed jars. A display of seeds from crops grown in North Dakota in the late 1890s.

will impinge on the lives of many future generations. This view ties in with the notion of intergenerational equity that has been popularized through the discourse on sustainable development. However, Wells does not engage the challenges of alleviating poverty, nor does he consider pathways to improve global development indicators such as education or access to basic health facilities. The solution he offers at the end of the book is that the West should "want less." No doubt this is admirable advice to the affluent, but how would it be applied to the indigent in the developing world? While acknowledging that a return to subsistence is not possible, he provides little guidance

on how to feed the world's growing population without relying on advanced agricultural innovation. As the director of the National Geographic Society's project on tracing human migration flows (the Genographic Project), Wells has a grasp of many disparate fields. Nonetheless, he seems to have been selective in his coverage to boost his argument. Ecological resilience and human adaptation receive scant attention, and Oriental and Occidental cultures are portrayed in stylized and static forms.

Wells concludes with a call for a shift from "logos" to "mythos," which has literary appeal but very little tangible currency. Instead, perhaps his goals would have been better served by focusing on a shift in "ethos"—cultural attributes, coupled with a quest for innovation. *Pandora's Seed* cultivates curiosity about the complex and chaotic linkages between human agency and nature but then implodes with an elegant yet simplistic argument.

References

1. J. Diamond, *Collapse: How Societies Choose to Fail and Succeed* (Viking, New York, 2005); reviewed in (5).
2. J. A. Tainter, *The Collapse of Complex Societies* (Cambridge Univ. Press, Cambridge, 1988).
3. C. Ponting, *A Green History of the World* (Sinclair-Stevenson, London, 1991).
4. M. Aldenderfer, N. M. Craig, R. J. Speakman, R. Popelka-Filcoff, *Proc. Natl. Acad. Sci. U.S.A.* **105**, 5002 (2008).
5. T. Flannery, *Science* **307**, 45 (2005).

The reviewer is at the Rubenstein School of Environment and Natural Resources, University of Vermont, 153 South Prospect Street, Burlington, VT 05401, USA. E-mail: saleem@alum.mit.edu

10.1126/science.1192608

Prepublication Data Release, Latency, and Genome Commons

Jorge L. Contreras

Researchers must disclose their data in order to achieve recognition and to enable others to test, validate, and challenge their hypotheses. In doing so, they create bodies of shared knowledge that are analogous to traditional public resources, such as forests and freeways, often referred to as “commons” (1, 2). The rate at which data are added to these information commons, however, varies greatly. The traditional practice has been to contribute experimental and observational data to the commons when, or shortly after, the analysis of that data is published, sometimes years after its initial collection (3, 4). Because of busy schedules, competitive pressures, and other interpersonal vagaries, the sharing of scientific data can be inconsistent even after publication (5, 6). Many traditional data-sharing practices were challenged, with significant and lasting effect, during the race to sequence the human genome.

The Bermuda Principles

In 1992, shortly after the initiation of the international Human Genome Project (HGP), the U.S. National Institutes of Health (NIH) and Department of Energy (DOE) adopted a policy requiring that genomic data be released within 6 months after generation (7), a substantial reduction from the typical 12- to 18-month period required by other government-funded projects (3, 8). But by 1996, leaders of the HGP realized that even 6 months was too slow for the massive project. They convened in Bermuda and agreed that all HGP sequence data be released to public databases (e.g., NIH’s GenBank), without restrictions on subsequent publication, within 24 hours after generation (9).

At a practical level, the so-called “Bermuda Principles” were necessary to coordinate activities of more than a thousand HGP researchers around the world. But other motivations were also at work. To many researchers, includ-

ing some HGP leaders, the human genome sequence was fundamentally different from other large data sets: It represented the common heritage of the human species and should not be encumbered by patents, a growing concern in the mid-1990s (10–12). By requiring rapid, prepublication data release of the human genome sequence, the ability of both the publicly funded data generators and others to patent the resulting data would be limited (13–15). And, whereas delays built into previous policies gave data generators time to analyze results and prepare papers before the data became available to others, the Bermuda organizers sacrificed investigators’ publication “priority” in the service of these other program goals.

Latencies: Knowledge and Rights

The framework for the information commons established by the Bermuda Principles and subsequent policies in the genome sciences can be thought of in terms of built-in temporal “latencies.” “Knowledge latency,” the period between the generation of a particular datum and its mandated release to the public commons (16), is immediately followed by “rights latency,” the period between the entry of a datum into the commons and the lifting of substantial encumbrances on its use, such as those imposed by policy or contract.

Latency characteristics of U.S. genomic data release policies (17) have evolved since Bermuda (see the figure below). A number

Scientific information commons can be modulated by adjusting the twin “dials” of knowledge and rights latency.

of factors contribute to the changes in these policies. For example, (i) concerns about academic recognition and tenure “credit” have led to a greater demand for attribution among data generators and the desire for at least some priority in preparing publications based on “their own” data (18, 19). (ii) Investigators whose work is driven primarily by the generation and testing of hypotheses have proven to be less comfortable with rapid, prepublication data release than investigators whose work has been driven primarily by the generation of large data sets (18–20). And (iii), issues regarding patenting of genetic material have taken on increasing prominence, together with the recognition by U.S. funding agencies that their ability to impose outright prohibitions on patenting by grant recipients is limited by the Bayh-Dole Act of 1980 (21, 22).

To address these issues, genomics policy-makers, after experimenting with a variety of less-specific directives (23), began to adapt latency mechanisms to dictate how and when data should be released and used (25). Distinct approaches soon emerged, employing latency-based rules to promote policy goals (e.g., access to data to promote scientific advancement, publication priority to provide incentives and to reward researchers, and minimization of encumbrances on the use of data once entered into the commons). But the approaches prioritized these goals differently.

A path taken by NIH is exemplified by the 2007 policy adopted for all NIH-funded genome-wide association studies (GWAS) (26) (see the figure). Rapid release of federally funded GWAS data is required, but data users must refrain from publishing or presenting related results during an “embargo” period of up to 12 months. This embargo, which may be implemented via “click-through” agreements or funding policy obligations (27), gives data generators a “head start” on preparing publications based on their data, yet data are still broadly available for the general advancement of science. Because the Bayh-Dole Act limits the government’s ability

LATENCY VARIABLES IN GENOMIC DATA RELEASE POLICIES

Genomics agreement	Year	Knowledge latency	Rights latency	References
<i>Funded by the U.S. federal government</i>				
NIH-DOE Guidelines	1992	6 months	0	(6)
Bermuda Principles	1996	24 hours	0	(8)
International HapMap Project	2003	Short	0	(38,39)
GAIN	2006	Short	9 months	(40)
NIH GWAS Policy	2007	Short	12 months	(26)
Encode + modENCODE	2008	Short	9 months	(41)
<i>Nongovernmental</i>				
SNP Consortium	1998	3 months	0	(42)
International SAE Consortium	2007	12 months	9 months	(28)

Latency variables in genomic data release policies. Policies designated “short” do not specify an exact timeline for data release but do require that data be released as rapidly as possible after generation or validation.

School of Law, Washington University in St. Louis, St. Louis, MO 63130, USA. E-mail: jlcontreras@wulaw.wustl.edu

to impose restrictions directly on patenting, this rapid data release requirement presents a means to frustrate patents that might otherwise restrict use of the data.

An alternate approach has been taken by privately organized groups such as the International Serious Adverse Events Consortium (iSAEC). The consortium establishes a holding period of up to 12 months before data are made publicly accessible, during which data generators can exclusively analyze and prepare papers (28). Compared with the NIH GWAS policy, this approach offers a stronger preservation of data generator publication priority, but at the expense of overall scientific advancement (as the data are not available for public use during the 12-month priority period). Patent issues are addressed via a separate “defensive patenting” policy [a strategy not available to governmental agencies (29)], under which the consortium files patent applications on discoveries, thus establishing an early “priority date,” but will subsequently contribute these rights to the public.

Beyond Genomics

The latency-based framework derived from genome commons policies has broader applicability to other scientific disciplines. Scientific information commons are under discussion in areas ranging from microbiology (30) to global climate change (31) to molecular chemistry (32). Policy designers in these fields cannot adopt wholesale the approaches taken in the genome commons. For example, whereas it may have been appropriate for genome commons policies to limit patent encumbrances on human DNA sequence data, such an approach may be inappropriate for other data sets, such as proprietary chemical compounds. Thus, the unique attributes, history, and norms of a given discipline must shape its data-sharing paradigms.

Issues associated with science commons have also been highlighted by the debate over open-access publishing and the potential release of vast quantities of published and unpublished scientific information to the public. As with genome commons, policy-makers negotiating the contours of open-access databases have already begun to rely on timing mechanisms to balance the competing policy objectives of scientists, government, and publishers (33, 34). The key to developing successful information commons is striking the appropriate balance among these competing objectives. Commons weighted too heavily in favor of data users are not likely to attract sufficient contributions from data generators,

whereas commons weighted too heavily in favor of data generators may not optimally advance the interests of science or the public. Thoughtful setting of temporal latency variables will result in scientific information commons that attract optimal quantities of data and thereby serve the overall advancement of science.

References and Notes:

- C. Hess, E. Ostrom, *Understanding Knowledge as a Commons: From Theory to Practice* (MIT Press, Cambridge, 2007).
- M. J. Madison, B. M. Frischmann, K. J. Strandberg, *Cornell Law Rev.* **95**, 657 (2010).
- National Research Council, *Bits of Power: Issues in Global Access to Scientific Data* (National Academy Press, Washington, DC, 1997).
- The optimal structure of publication-based data release is itself a large and contentious issue that overlaps with, but is beyond the scope of, this paper on prepublication data release. See, e.g., (35).
- D. Blumenthal *et al.*, *Acad. Med.* **81**, 137 (2006).
- E. G. Campbell *et al.*, *JAMA* **287**, 473 (2002).
- DOE, *Human Genome News*, January 1993, p. 4.
- National Academy of Sciences, *Ensuring the Integrity, Accessibility, and Stewardship of Research Data in the Digital Age* (National Academy Press, Washington, DC, 2009).
- Summary of principles agreed at the International Strategy Meeting on Human Genome Sequencing, Bermuda, 25 to 28 February 1996; www.ornl.gov/sci/techresources/Human_Genome/research/bermuda.shtml#1.
- National Research Council, *Mapping and Sequencing the Human Genome* (National Academy Press, Washington, DC, 1988).
- F. Collins, *The Language of Life* (HarperCollins, New York, 2010).
- J. D. Watson, *Science* **248**, 44 (1990).
- International Human Genome Sequencing Consortium, *Nature* **409**, 860 (2001).
- National Research Council, *Reaping the Benefits of Genomic and Proteomic Research* (National Academies Press, Washington, DC, 2006).
- Releasing data early prevents the data generator from patenting it (there is a statutory ban on patenting material that one has previously released to the public) and also prevents others from patenting it (as the early release acts as “prior art,” limiting subsequent claims of “novelty”).
- Most “publicly accessible” genomic and phenotypic data residing in databases such as NIH’s Database of Genotypes and Phenotypes (dbGaP) are actually made available only to verified scientific researchers (whether commercial or academic) and not to the public at large. Even under the most stringent rapid data release policy designs, practical, technological, and personnel issues may bear on the usability and effectiveness of data available in the commons.
- Detailed data release policies exist for non-U.S. genomics projects (e.g., the Wellcome Trust and Medical Research Council in the U.K. and Genome Canada). This article focuses on U.S. policies because they have made great use of the timing mechanisms that are the subject of this analysis. The reason for this may be the lesser prevalence and enforcement of bioscience patents outside the United States, as the laws of those jurisdictions are less amenable to patent filings on genomic discoveries.
- Toronto International Data Release Workshop Authors, *Nature* **461**, 168 (2009).
- J. Kaye, C. Heeney, N. Hawkins, J. de Vries, P. Boddington, *Nat. Rev. Genet.* **10**, 331 (2009).
- National Human Genome Research Institute (NHGRI), NIH, “Policy for release and database deposition of sequence data” (NHGRI, Bethesda, MD, 21 December 2000); www.genome.gov/10000910.
- A. K. Rai, R. S. Eisenberg, *Law Contemp. Probl.* **66**, 289 (2003).
- ENCODE Project Data Release Policy (2003–2007), www.genome.gov/12513440.
- For example, the accord reached at a 2003 summit in Ft. Lauderdale provides for no formal restrictions on the use of data but requests that users “act responsibly to promote the highest standards of respect for the scientific contribution of others” (24).
- Wellcome Trust, Sharing Data from Large-Scale Biological Research Projects: A System of Tripartite Responsibility, meeting, Ft. Lauderdale, FL, 14 to 15 January 2003; www.genome.gov/Pages/Research/WellcomeReport0303.pdf.
- The temporal mechanisms described in this paper are not the only means that have evolved to address the issue of data generator priority. Other techniques include explicit agency guidelines requiring the acknowledgment of relevant data generators and the early publication by data generators of so-called “statements of intent” and “marker papers.”
- NIH, Policy for sharing of data obtained in NIH supported or conducted genome-wide association studies (GWAS). *Fed. Regist.* **72**, 49,290 (2007).
- Both the enforceability and actual enforcement of these contractual mechanisms are uncertain. Absent a strong trend toward underenforcement, which has not been observed, it is useful to analyze these policy timing mechanisms at face value, without accounting for under-enforcement by rights holders.
- International SAE Consortium (iSAEC), Intellectual Property Program, www.saeconsortium.org/?q=node/12.
- Because iSAEC is not directly regulated by the Bayh-Dole Act that limits the government’s ability to restrict patents, it has the liberty to modify the patenting process directly, independently of latency mechanisms. Such consortia can thus use timing mechanisms in a more focused manner, without regard for patent concerns, to better address other priorities (e.g., they do not have to rely on the suboptimal embargo approach (27) to protect data generators’ publication priority).
- C. Hess, E. Ostrom, *Int. Soc. Sci. J.* **58**, 335 (2006).
- O. Heffernan, *Nature Online*, 4 September 2009.
- G. Brumfiel, *Nature* **453**, 139 (2008).
- NIH, “Revised policy on enhancing public access to archived publications resulting from NIH-funded research” (NOT-OD-08-033, NIH, Bethesda, MD, 7 April 2008).
- The NIH open-access policy (33) gives scientific journals a 1-year “head start” before journal articles are released to public governmental databases. Legislation proposed in the U.S. Congress (36) would expand the NIH’s open-access mandate to all federal agencies, with a shortened embargo period of 6 months. Legislation opposing both initiatives has also been proposed in Congress (37).
- P. N. Schofield *et al.*, *Nature* **461**, 171 (2009).
- Federal Research Public Access Act (FRPAA), H.R. 5037 (2010).
- Fair Copyright in Research Works Act, H.R. 801 (2010).
- International HapMap Consortium, *Nature* **426**, 789 (2003).
- International HapMap Project, Data Release Policy, www.hapmap.org/datareleasepolicy.html.
- Genetic Association Information Network (GAIN), Data Use Certification Agreement (version of 3 December 2008); http://dbgap.ncbi.nlm.nih.gov/aa/wga.cgi?view_pdf&stacc=phs000016.v1.p1.
- NHGRI, “ENCODE consortia data release, data use, and publication policies” (NHGRI, Bethesda, MD, 2008); www.genome.gov/Pages/Research/ENCODE/ENCODEDataReleasePolicyFinal2008.pdf.
- A. Holden, *Biotechniques* **32**, S22 (2002).
- J.L.C. is a member of the National Advisory Council for Human Genome Research, the oversight body for NHGRI, NIH; is a paid consultant to the International SAE Consortium Ltd.; and has served as a paid consultant to the SNP Consortium Ltd.

10.1126/science.1189253

PLANT SCIENCE

Launched at 36,000g

Johan L. van Leeuwen

Most mosses, including peat mosses of the genus *Sphagnum* (about 285 species), disperse their spores by turbulent wind (1). In still air, spores (22 to 45 μm in size) sink at only 0.5 to 2 cm/s, ideal for wind dispersal (2). However, spore capsules, positioned on a short stalk, grow to heights of about 1 cm and do not extend into the atmospheric turbulent boundary layer (more than 10 cm above ground). Peat mosses solve this problem with an “air gun” mechanism that explosively discharges spores from a pressurized cylindrical capsule ~ 2 mm in length (see the figure), projecting spores over 10 to 20 cm (1–3). On page 406 of this issue, Whitaker and

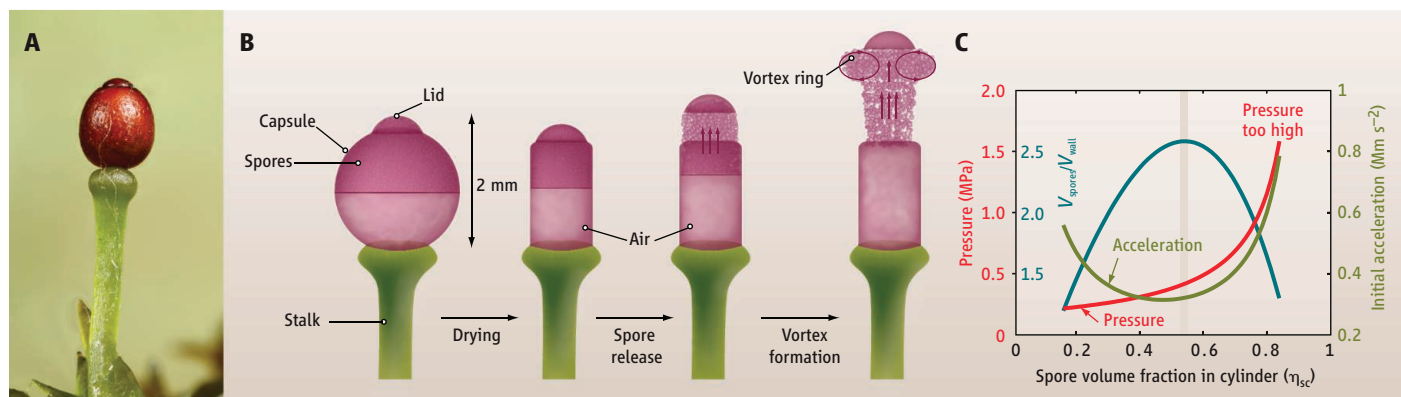
tial and even turbulent flow regime, enabling spores to travel long distances by entering the dynamics of a larger-scale vortex ring.

The power for the explosion stems from compressed air in the spore capsule. The intact mature capsule in peat mosses is spherical when wet. The upper portion of the capsule holds 20,000 to 240,000 spores (5), whereas the middle and lower portions contain air. As water evaporates from the epidermis through pores, the capsule transforms into a cylindrical shape of approximately the same height (1, 4, 6). The epidermal cells shrink, thus decreasing the circumference of the capsule. The final volume of the cylindrical capsule is only about three-eighths of

Peat mosses project thousands of spores in a turbulent vortex ring from a millimeter-sized pressurized cylindrical capsule.

the spores and traveling distance rises with increasing pressure and spore mass.

However, there is a price to pay for increased spore content, because the mechanical stress in the capsule wall is proportional to the internal pressure and inversely proportional to the wall thickness. Hence, wall thickness needs to increase linearly with pressure and thus nonlinearly with η_{sc} if wall stress is kept constant, which seems reasonable for identical material properties and a fixed safety factor for rupture. The required ratio of spore/wall volume (see the figure) has a skewed parabolic shape and peaks at about 2.6 for $\eta_{\text{sc}} = 0.54$, which is close to observed values of Whitaker and Edwards, and others (3, 4, 7). The multifaceted shape of



Spore discharge. (A) Spore capsule of *Sphagnum fimbriatum* on a short stalk. (B) The wet spherical capsule becomes cylindrical by drying. Quick release of the lid triggers spore discharge by internal air pressure. The jet of spores and air rolls up into a turbulent ring vortex that carries spores up to 15 to 20 cm. (C) Air pressure

(above ambient) rises nonlinearly with the volume fraction of spores (η_{sc}). Initial spore acceleration is highest for both low and high η_{sc} because of low spore mass or high pressure. $V_{\text{spores}}/V_{\text{wall}}$ is the spore/wall volume ratio. Vertical tan line corresponds to the predicted optimum in spore content, similar to observed η_{sc} (2–4).

Edwards (4) report that an upward-traveling turbulent vortex ring of spores and air is formed by this explosion within less than 0.2 ms, carrying sufficient momentum to reach the turbulent boundary layer.

The upward momentum of the air and spore mass determines the distance traveled by the spores. Whitaker and Edwards point out that the collective discharge of many spores is required to tip the aerodynamic balance from friction forces to inertial forces. A single spore projected at the same speed would decelerate so fast by viscous drag that it would travel only a few millimeters. The collective discharge of spores scales up the dynamics into the iner-

the original one, a reduction accomplished mainly by air compression. The change into an upright pressurized cylinder (see the figure) promotes vertical launch of the spores.

The volumes of air in the spherical (V_0) and cylindrical (V_1) states can be computed from the volume fraction of the spores (η_{sc}) and other tissues in the cylinder. Hence, air pressure in the cylinder (p_1) can be derived from Boyle's law ($p_0 V_0 = p_1 V_1 = \text{constant}$), setting p_0 equal to the ambient pressure. Even with the assumed isothermal conditions and taking $\eta_{\text{sc}} = 0.53$, as observed for *Sphagnum*, a pressure (relative to ambient) of about 400 kPa (5 atm absolute pressure) is estimated, which is similar to previous measurements (3). Air volume in the cylinder drops linearly with η_{sc} , but air pressure rises nonlinearly with η_{sc} . The momentum of

the spores promotes packing efficacy while reducing the sinking speed in air (relative to spheres of similar size).

The spore velocities recorded by Whitaker and Edwards (which was time-resolved with ultrahigh-speed videos of 10^4 to 10^5 frames per second) were as high as 30 m/s. Such velocities require very high accelerations because the travel distance within the tiny capsule is only a millimeter or less. The lid is presumably released through a combination of tension in the capsule wall (6, 7) and internal pressure, causing the sudden rupture of the relatively weak epidermal attachment ring, and enabling the upward acceleration of the spore mass by the vertical force of the compressed air (equal to air pressure times transverse area of the cylinder). Assuming that the compacted spores move initially as

one mass and applying Newton's second law (force equals mass times acceleration), one can calculate an initial acceleration of about $320,000 \text{ m/s}^2$ or $32,000g$ for a realistic η_{sc} of 0.54 and a density of the spores of 1200 kg/m^3 . Higher values of η_{sc} would lead to even higher accelerations. Whitaker and Edwards' movie S3 shows that the spore cloud progresses from rest by about 1.8 mm in 0.1 ms, representing an average speed of 18 m/s. If velocity increases linearly from rest, the actual speed is 36 m/s after 0.1 ms, resulting in an estimated acceleration of $360,000 \text{ m/s}^2$, 12.5% above the calculation.

The energy stored in the compressed air (about 0.27 mJ for $\eta_{sc} = 0.54$) is only partly

used to accelerate spores. As long as the air expands within the capsule, this energy mainly converts into kinetic energy of the spores. When most spores have left the capsule, relatively more energy converts into air convection, heat, and audible sound (2).

The upscaling principle elucidated by Whitaker and Edwards for peat moss has been exploited abundantly in nature for the dispersal of microscopic items, whether reproductive or waste products. Examples are found in other mosses, fungi (1), flowering plants [pollen dispersal (8)], and microscopic waste dispersal through water jets in filtering sponges and tunicates. We are only starting to discover and appreciate the wide array of unique solu-

tions devised by nature to enhance the dispersal of microscopic particles.

References

1. C. T. Ingold, *Spore Liberation* (Clarendon, Oxford, 1965).
2. S. Sundberg, *Ann. Bot.* **105**, 291 (2010).
3. S. Nawaschin, *Flora* **83**, 151 (1897).
4. D. L. Whitaker, J. Edwards, *Science* **329**, 406 (2010).
5. S. Sundberg, H. Rydin, *J. Bryol.* **20**, 1 (1998).
6. J. G. Duckett, S. Pressel, K. M. Y. P'ng, K. S. Renzaglia, *New Phytol.* **183**, 1053 (2009).
7. S. Sundberg, *New Phytol.* **185**, 886 (2010).
8. J. Edwards, D. Whitaker, S. Klionsky, M. J. Laskowski, *Nature* **435**, 164 (2005).

Supporting Online Material

www.sciencemag.org/cgi/content/full/329/5990/395/DC1
SOM Text

10.1126/science.1193047

PHYSICS

Pairs Rule Quantum Interference

James D. Franson

Quantum interference is one of the most mysterious features of quantum mechanics. In fact, Feynman referred to the double-slit interference experiment for single particles as the “only” mystery in quantum mechanics (1). On page 418 of this issue, Sinha *et al.* (2) describe a recent experiment that shows that quantum interference from a single photon arises only from pairs of possible paths through an interferometer. There is no need to invoke additional interference terms that might arise from interactions of three or more paths.

In classical mechanics, outcomes can be described directly with probabilities. In quantum mechanics, the probability of an outcome is obtained from probability amplitudes (wave functions) that may be negative or even take complex values. For example, if a single photon can traverse three possible paths through an interferometer to reach a detector, the different paths have probability amplitudes ψ_1 , ψ_2 , and ψ_3 (see the left-hand panel of the figure). The total probability amplitude ψ for the photon to reach the detector is the sum $\psi = \psi_1 + \psi_2 + \psi_3$, but this is not the probability P that the photon will reach the detector. Probability is calculated by taking the square of the probability amplitude as

$$P = |\psi|^2 = \psi^* \psi = |\psi_1 + \psi_2 + \psi_3|^2$$

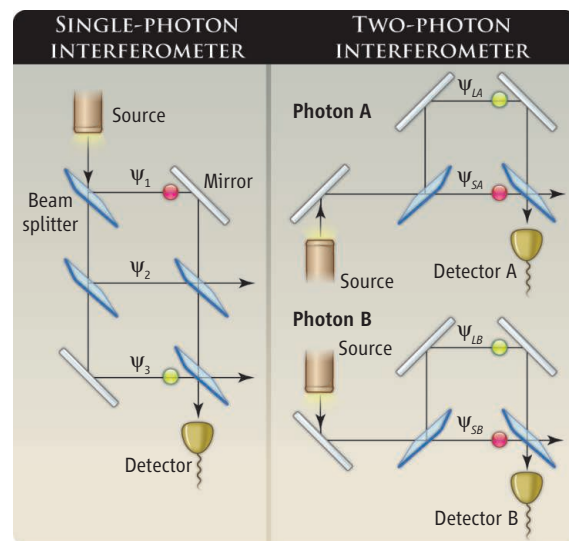
(the magnitude of the complex number ψ

is obtained by multiplying it by its complex conjugate ψ^*). Unlike the classical situation, where the individual probabilities would simply add, probability amplitudes can add constructively or cancel destructively. These interference effects depend on the phase shifts encountered in each path.

If a measurement had been made, it would have revealed which one of the three paths the photon took while it was traversing the interferometer. Nevertheless, a single photon must somehow determine the phase shift in all three paths to give the correct interference effects. This is the fundamental “mystery”

referred to by Feynman (1). The product of ψ^* and ψ is a series of terms of the form $\psi_i^* \psi_j$, so the detection probability should be determined by interference only between all possible pairs of paths through the interferometer (2). An example of such a pair of paths is indicated in the panel, left of the figure by the red and yellow dots, one of which comes from ψ^* and the other from ψ .

Sinha *et al.* performed a careful series of measurements in which there were three possible paths that a single photon could take through an interferometer. By blocking off various combinations of paths, they measured the contributions from all possible pairs of paths, which were found to contribute at least 99% of the total detection probability. This sets an upper limit of approximately 1% for any contribution from three or more paths, which is consistent with their experimental error. They performed the measurements using true single



Pairwise through many paths. (Left) A single-photon interferometer consists of a source, mirrors, beam splitters, and a detector. Quantum interference only occurs between pairs of optical paths, such as those labeled by the red and yellow dots, as verified in a recent experiment by Sinha *et al.* (Right) A two-photon interferometer (3) is depicted in which quantum interference can occur between sets of four optical paths (where the subscripts L and S refer to long and short paths taken by photons A and B). Quantum interference effects arise regardless of the distance between the interferometers.

Physics Department, University of Maryland Baltimore County, Baltimore, MD 21250, USA. E-mail: jfranson@umbc.edu

photons from a nonclassical source, as well as using weak laser pulses with an average occupation of much less than one photon.

These ideas can be generalized to two-photon interferometers (3, 4), where there will be interference between sets of four optical paths. This case is illustrated with the two-photon interferometer in the right-hand panel of the figure (3), where a pair of entangled photons (ones that are nonclassically correlated) pass through two distant interferometers. The photons are known to be emitted at the same time, but that time is uncertain in the quantum-mechanical sense (a superposition state). If both photons are detected at the same time, they must have traveled the same distance because they were emitted simultaneously. Thus, there is a probability amplitude ψ_{LA} ψ_{LB} that both photons took the longer paths and another probability amplitude ψ_{SA} ψ_{SB} that both photons took the shorter paths.

The square of the sum of these probability amplitudes now involves interference between four optical paths, such as

$$\psi_{LA}^* \psi_{LB}^* \psi_{SA} \psi_{SB},$$

as illustrated once again by the red and yellow dots. The probability that photon A will be detected depends not only on the phase shifts in the interferometer that it passed through but also on the phase shifts in the distant interferometer. Any classical explanation of these results would require information to be transmitted faster than the speed of light c (3); indeed, a recent experiment showed that the information transfer would need to be four orders of magnitude faster than c (5). This situation does not violate special relativity because the photon detection outcomes cannot be controlled by an experimenter to send messages faster than c . Multiparticle interference of this kind only involves interference between pairs of more complicated probability amplitudes and could be tested in future experiments.

The development of a theory that combines quantum mechanics with general relativity is one of the major research goals of modern physics. If gravity can be quantized, it would be possible in principle to perform quantum interference experiments

similar to those of the figure with gravitons instead of photons. The unification of these two fundamental theories is complicated by a number of factors, including the fact that summing up all of the possible probability amplitudes gives infinite results that are nonphysical—that is, mathematical singularities in the theory do not appear to be renormalizable to finite values. Small modifications to quantum mechanics may be necessary to achieve a consistent theory of quantum gravity, and experiments of this kind may be useful in setting limits on what modifications are allowed.

References

1. R. P. Feynman, R. B. Leighton, M. Sands, *The Feynman Lectures on Physics* (Addison Wesley, Reading, MA, 1964).
2. U. Sinha, C. Couteau, T. Jennewein, R. Laflamme, G. Weihs, *Science* **329**, 418 (2010).
3. J. D. Franson, *Phys. Rev. Lett.* **62**, 2205 (1989).
4. M. A. Horne, A. Shimony, A. Zeilinger, *Phys. Rev. Lett.* **62**, 2209 (1989).
5. D. Salart, A. Baas, C. Branciard, N. Gisin, H. Zbinden, *Nature* **454**, 861 (2008).

10.1126/science.1192624

MEDICINE

Wnt Fans the Flames in Obesity

Da Young Oh and Jerrold M. Olefsky

Obesity is linked to major adverse health outcomes such as insulin resistance and type 2 diabetes (1). The mechanisms underlying insulin resistance in this state are multiple and complex, including the recruitment of immune cells, particularly macrophages, to adipose tissue. This results in chronic, low grade inflammation that is causally associated with insulin resistance. With obesity, adipose tissue mass expands and adipocyte (fat cell) size increases. Collectively, adipocytes constitute the body's largest endocrine organ, producing an array of peptide hormones called adipokines (2). On page 454 of this issue, Ouchi *et al.* (3) report that a protein secreted by adipocytes acts as an anti-inflammatory adipokine, restraining the chronic inflammatory state and consequently improving insulin sensitivity.

Adipocytes respond to an increase in adipose tissue mass by secreting chemokines that attract monocytes (4, 5). These

then become activated proinflammatory adipose tissue macrophages that secrete cytokines which work locally through paracrine mechanisms to cause decreased sensitivity to insulin in nearby adipocytes (6). The macrophages also secrete chemokines that recruit further waves of monocytes into the adipose tissue, creating a feed-forward chronic proinflammatory state. The general outline of this scenario is well recognized, but the mechanisms underlying this multi-step process are not completely understood.

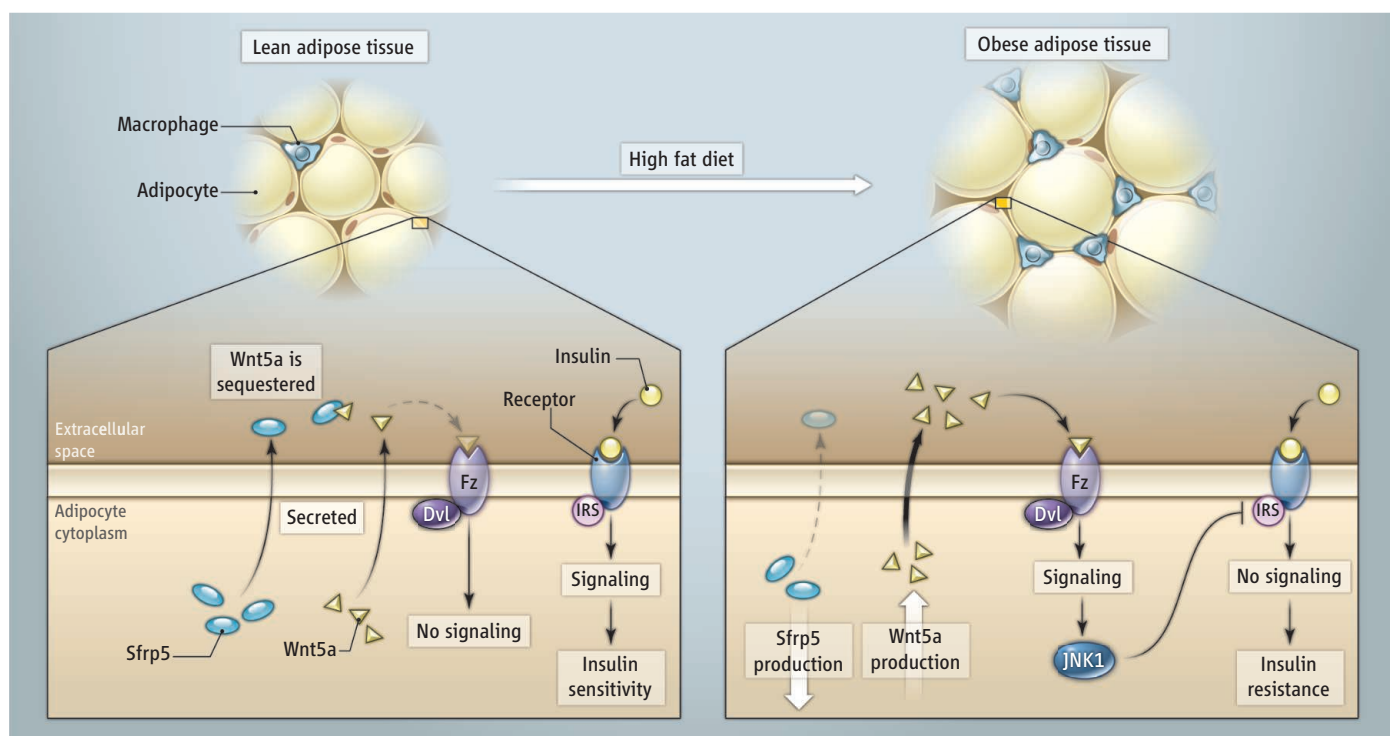
Ouchi *et al.* now incorporate the function of the cellular Wnt signaling pathway in macrophages and adipocytes into this scenario. The Wnt proteins regulate cell proliferation, differentiation, and developmental processes in many organisms. They are a family of secreted glycoproteins that signal through a cell surface receptor called frizzled (Fz) to stimulate either a noncanonical or canonical signaling pathway (7) (see the figure). The canonical pathway stabilizes the cytoplasmic protein β -catenin, leading to increased transcription of target genes. The noncanonical pathway stimulates guanosine triphosphatases (RhoA and Rac1), leading

The balance between two factors released by mammalian fat cells controls the degree of inflammation and insulin sensitivity in adipose tissue.

to activation of the serine-threonine kinase c-Jun N-terminal kinase 1 (JNK1).

Ouchi *et al.* show that Wnt5a, which stimulates the noncanonical pathway, is expressed in mammalian adipose tissue. Its expression in mice was induced by a high fat diet and obesity. In mouse immune cells, Wnt5a activated JNK1, which triggered a proinflammatory response. In insulin target cells, such as adipocytes, activated JNK1 impairs the activity of a target protein called insulin receptor substrate-1 (IRS-1), leading to decreased insulin signaling and the development of insulin resistance (8). Using gene array analyses, Ouchi *et al.* found that expression of the secreted frizzled-related protein five (Sfrp5) is high in mouse adipocytes, but is decreased in various rodent models of obesity. Sfrp5 acts as a decoy receptor that binds and sequesters Wnt5a in the extracellular environment, thus preventing activation of Fz and attenuating noncanonical Wnt signaling. Therefore, the obesity-induced decrease in Sfrp5 production by adipocytes, along with increased Wnt5a expression, should lead to enhanced inflammatory signaling and insulin resistance,

Department of Medicine, Division of Endocrinology and Metabolism, University of California, San Diego, La Jolla, CA 92093, USA. E-mail: jolefsky@ucsd.edu



Adipocyte rheostat. Wnt5a stimulates the noncanonical Wnt signaling pathway in adipocytes by binding to frizzled (Fz), which stimulates a pathway that activates dishevelled (Dvl) and JNK1. Sfrp5 is secreted from adipocytes and sequesters Wnt5a, preventing pathway activation of this pathway. This allows adipocytes to respond

to insulin. In the obese state, adipocytes enlarge and secrete more Wnt5a, but less Sfrp5. This allows activation of the noncanonical Wnt pathway, which blocks insulin signaling and causes insulin insensitivity. Macrophages present in obese adipose tissue also respond to Wnt5a and produce proinflammatory cytokines.

precisely the situation in obesity.

Ouchi *et al.* also observed that when put on a high fat diet, mice genetically engineered to lack Sfrp5 showed greater adipose tissue inflammation and insulin resistance due to unrestrained Wnt5a activity. By contrast, overexpression of Sfrp5 in adipocytes of these animals blocked Wnt5a activity and prevented the inflammatory, insulin resistant state. Likewise, systemic administration of Sfrp5 into animals lacking Sfrp5 improved the overall state of glucose intolerance and insulin sensitivity and diminished inflammation. Overall, these studies make a strong argument that the balance between Wnt5a and Sfrp5 expression serves as a rheostat to control the degree of noncanonical Wnt signaling in adipose tissue macrophages and adipocytes, which, in turn, modulates inflammation and the state of insulin sensitivity.

Ouchi *et al.* also probed the cellular mechanisms underlying these effects and determined that JNK1 is a control point for Wnt signaling input to inflammatory and metabolic events. When the authors treated mouse macrophages with a small molecule JNK1 inhibitor, the stimulatory effect of Wnt5a on proinflammatory cytokine release was blocked. Furthermore, a lack of JNK1 in the Sfrp5-null mice reversed the effect of

Sfrp5 depletion to promote inflammation and insulin resistance.

The concept that noncanonical Wnt signaling promotes a proinflammatory state in adipose tissue adds a new layer to our understanding of obesity and raises several questions. Activation of the noncanonical Wnt pathway in various cell types has pleiotropic effects on many different biological processes, including cell adhesion, migration, actin cytoskeletal organization, lymphopoiesis, and inflammation. In adipose tissue of obese individuals, is the increased signaling by this pathway restricted to inflammation or does it link to these other processes as well? The insulin resistance of obesity and type 2 diabetes is systemic, involving liver and skeletal muscle, in addition to adipose tissue. One question is whether noncanonical Wnt signaling in muscle and/or liver could interact with the insulin signaling pathway in these tissues. Similarly, does systemic administration of Sfrp5 block noncanonical Wnt signaling directly in muscle and liver, or are its effects to improve insulin sensitivity in these tissues indirect?

Stepping back from the more detailed mechanisms, there are general questions that now arise from the observations of Ouchi *et al.* How do proinflammatory events in adipose tissue communicate with

the other major insulin target tissues, muscle and liver, to establish a state of systemic insulin resistance? What is the teleologic purpose of immune cell invasion into adipose tissue during the course of obesity? This understanding will guide the development of therapeutics, which could include harnessing the insulin sensitizing effects of Sfrp5 to treat obesity-related insulin resistance. Such approaches are greatly needed as there is now an enormous health burden in the United States (9) and other countries brought about by the rising incidence of obesity, type 2 diabetes, and associated metabolic diseases.

References and Notes

1. S. E. Shoelson, L. Herrero, A. Naaz, *Gastroenterology* **132**, 2169 (2007).
2. M. E. Trujillo, P. E. Scherer, *Endocr. Rev.* **27**, 762 (2006).
3. N. Ouchi *et al.*, *Science* **329**, 454 (2010); published online 17 June 2010 (10.1126/science.1188280).
4. S. P. Weisberg *et al.*, *Invest.* **112**, 1796 (2003).
5. H. Xu *et al.*, *Invest.* **112**, 1821 (2003).
6. S. Schenk, M. Saberi, J. M. Olefsky, *J. Clin. Invest.* **118**, 2992 (2008).
7. F. J. Staal, T. C. Luis, M. M. Tiemessen, *Nat. Rev. Immunol.* **8**, 581 (2008).
8. V. Aguirre, T. Uchida, L. Yenush, R. Davis, M. F. White, *J. Biol. Chem.* **275**, 9047 (2000).
9. A. H. Mokdad *et al.*, *JAMA* **282**, 1519 (1999).
10. Supported by NIH grants DK033651, DK074868, and DK063491.

10.1126/science.1193404

PHILOSOPHY OF SCIENCE

Machine Science

James Evans and Andrey Rzhetsky

Scientists today cannot hope to manually track all of the published science relevant to their work. A cancer biologist, for instance, can find more than 2 million relevant papers in the PubMed archive, more than 200 million Web pages with a Google search, and databases holding results from experiments that produce millions of gigabytes of data.

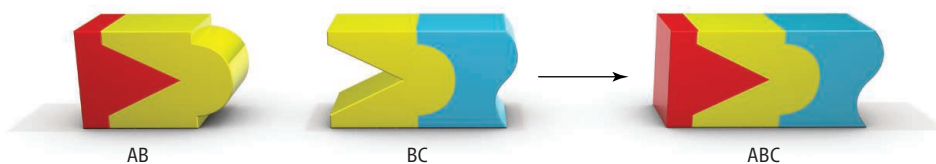
This explosion of knowledge is changing the landscape of science. Computers already play an important role in helping scientists store, manipulate, and analyze data. New capabilities, however, are extending the reach of computers from analysis to hypothesis. Drawing on approaches from artificial intelligence, computer programs increasingly are able to integrate published knowledge with experimental data, search for patterns and logical relations, and enable new hypotheses to emerge with little human intervention. Scientists have used such computational approaches to repurpose drugs, functionally characterize genes, identify elements of cellular biochemical pathways, and highlight essential breaches of logic and inconsistency in scientific understanding. We predict that within a decade, even more powerful tools will enable automated, high-volume hypothesis generation to guide high-throughput experiments in biomedicine, chemistry, physics, and even the social sciences (1).

Proponents of data-driven science (2–4) conjecture that hypotheses are obsolete: New knowledge will simply emerge from mechanical application of algorithms that mine data for plausible patterns. This approach is attractive, but there are potential pitfalls. The discovery of patterns from data alone is similar to the task faced by an explorer in an unfamiliar jungle, without a guide. With no sense of what is already known about the environment or its perils, she is likely to misclassify what she sees—fearing the intimidating but harmless snake; ignoring the tiny lethal frog.

Recent research demonstrates how scientists can use computers to become better-informed and more agile explorers. New computational tools can expand the pool of concepts and relations used for generating automated hypotheses by (i) drawing more

from the vast corpus of published science, and (ii) synthesizing new higher- and lower-order concepts and relations from the existing pool of knowledge. This approach can enable scientists studying a particular natural system, such as a biochemical pathway, to identify and fill in missing pieces, and traverse reasoning chains much longer than those possible with the unaided mind. For example, researchers have used computation to increase the number of candidate genetic aberrations considered in synthesizing hypotheses about disease (5–7). They have also increased the number of potential biological activities involved in describing new gene functions (8, 9) and ironed out past errors (10). Similarly, scientists have used computation to increase

Soon, computers could generate many useful hypotheses with little help from humans.



Logical leaps. Scientific knowledge and concepts can be represented as jigsaw puzzle pieces that, with the help of new computational tools, can be assembled into new hypotheses. In Swanson's ABC model, if the literature from one scientific subfield includes two concepts (A, red, and B, yellow), and the literature from another subfield includes B and C (blue), then an analyst may computationally infer that A and C are directly or indirectly related, potentially leading to new hypotheses that cross subfield boundaries.

the potential number of proteins and metabolites involved in biochemical networks, and to generate predictions about which locations in those networks could be altered to improve health (11) and to identify elements misidentified as participating in a network (12).

Merely increasing the pool of concepts and relations, however, would simply generate multitudes of low-quality hypotheses. Scientists can profitably restrict that multitude by using a selection process that draws on insights into the social, cultural, and cognitive production of science. For example, Swanson pioneered the ABC model of hypothesis generation, which focuses on hypotheses that cross boundaries between distinct scientific literatures. If concepts A and B are studied in one literature, and B and C in another, Swanson assumed transitivity to hypothesize that A implies C (see the figure and fig. S1). He then demonstrated that novel A-to-C inferences were likely to be true, although unlikely to be arrived at via other means (13–16). Through this approach, Swanson hypothesized that fish oil could lessen the symptoms of Ray-

naud's blood disorder and that magnesium deficits are linked to migraine headaches. This heuristic relies on an implicit understanding of scientific communities and publishing norms. It assumes that unpublished ideas within a research community are less valuable than ideas that link seemingly unrelated communities. Within a subfield, scientists are typically familiar with all of "their own" ideas, so unpublished connections more likely represent negative knowledge—superficially plausible ideas that participants know are wrong from experience. Unpublished ideas about subjects (such as the role of particular molecules or genes) that cross subfield boundaries, however, are much more likely to represent unasked questions. A recent analy-

sis of biomolecules common to several fields of biomedicine, for instance, suggests that many communities could profit from generating predictions that bridge field boundaries and link disparate properties of these molecules or other scientific concepts. (17).

Automated expansion of concepts and relations across community boundaries is severely constrained by incompatibilities in the language used by different scientific communities (18, 19). Because subfields have distinct histories, they often use different language to express the same concept, or similar terms to refer to unrelated entities. If researchers could computationally map these languages onto one another, as some are beginning to do with medical terminologies (20), they could vastly increase the number of possible hypotheses. Mapping concepts across languages would highlight parallels in theories from different domains, as well as changes in meaning with time (semantic drift) and multiple meanings (polysemy). These differences could be computationally mined to identify novel conceptual linkages.

By prioritizing hypotheses that contain concepts spanning existing scientific theories, languages, and cultures, investigators could productively focus on the most novel (21).

Analysts can also increase the pool of concepts and relations by computationally synthesizing new concepts and relations from those previously published. Computers have been deployed to “coarse grain” or identify new, higher-order aggregates of established concepts within studies of biological pathways, medical syndromes, and social classes (22). Scientists have also discovered new relations by identifying regular similarities between existing elements (23, 24). They have efficiently constricted the vast number of possible new aggregates by focusing on those that share physical properties or patterns, or integrate components of a broader system, such as a particular disease.

In the past, computational approaches have been more successful in small, well-

defined systems than in larger, less studied, or more complex ones. The explosion of data from high-throughput experiments, however, increasingly presents researchers with very complicated systems. Facing these data with questions equal in scale and complexity will be critical because, in the words of Mark Twain, “you can’t depend on your eyes when your imagination is out of focus” (25).

References and Notes

1. D. Lazer et al., *Science* **323**, 721 (2009).
2. L. Hood, *BioCommerce Week*, 9 September 2004.
3. T. Golub, *Nature* **464**, 679 (2010).
4. M. A. O’Malley, K. C. Elliott, C. Haufe, R. M. Burian, *Cell* **138**, 611 (2009).
5. C. Perez-Iratxeta, P. Bork, M. A. Andrade, *Nat. Genet.* **31**, 316 (2002).
6. I. Iossifov, T. Zheng, M. Baron, T. C. Gilliam, A. Rzhetsky, *Genome Res.* **18**, 1150 (2008).
7. J. Liu et al., *Science* **323**, 1218 (2009).
8. R. D. King et al., *Nature* **427**, 247 (2004).
9. R. D. King et al., *Science* **324**, 85 (2009).
10. J. Blake, personal interview, 5 February 2009.
11. D. A. Ruths et al., *J. Comput. Biol.* **13**, 1546 (2006).
12. T. L. Hsiao, et al., *Nat. Chem. Biol.* **6**, 34 (2010).
13. D. R. Swanson, *Perspect. Biol. Med.* **30**, 7 (1986).
14. D. R. Swanson, *Bull. Med. Libr. Assoc.* **78**, 29 (1990).
15. M. Weeber et al., *J. Am. Med. Inform. Assoc.* **10**, 252 (2003).
16. D. R. Swanson, N. R. Smalheiser, *Artif. Intell.* **91**, 183 (1997).
17. M. Cokol et al., *Nat. Biotechnol.* **23**, 1243 (2005).
18. Z. S. Harris, in *Sublanguage: Studies of Language in Restricted Semantic Domains*, R. Kittredge, J. Lehrberger, Eds. (de Gruyter, Berlin, 1982), pp. 231–236.
19. Z. S. Harris, *The Form of Information in Science: Analysis of an Immunology Sublanguage* (Kluwer Academic, Dordrecht, Netherlands, 1989).
20. The National Library of Medicine’s Metathesaurus MetaMap connects more than 100 distinct medical terminologies.
21. J. D. Wren, *BMC Bioinform.* **5**, 145 (2004).
22. J. Feret, V. Danos, J. Krivine, R. Harmer, W. Fontana, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 6453 (2009).
23. M. Schmidt, H. Lipson, *Science* **324**, 81 (2009).
24. J. Bongard, H. Lipson, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 9943 (2007).
25. M. Twain, *A Connecticut Yankee in King Arthur’s Court* (Webster, New York, 1889).

Supporting Online Material

www.sciencemag.org/cgi/content/full/329/5990/399/DC1
Fig. S1

10.1126/science.1189416

PLANETARY SCIENCE

Carbonates and Martian Climate

Ralph P. Harvey

The case for global climate change on Mars is neither ambiguous nor arguable. Cold, dry, and dusty right now, the surface of Mars shows ancient fluvial valley networks, catastrophic flood channels, sediment-filled basins, and many other features that require stable liquid water at the planet’s surface. Such observations from orbit (1) imply that ancient Mars was substantially warmer than it is today. The favored scenario to heat up a young Mars is greenhouse warming driven by a thick, primitive CO₂-rich atmosphere of the kind thought common to all the terrestrial planets (2). Clearly, the greenhouse phase didn’t last long for Mars. As a result, almost every model for martian climate history includes an appreciable drawdown of CO₂ by precipitation of carbonates. However, only a small fraction of the carbonates predicted by this theory have been detected. On page 421 of this issue, Morris et al. (3) report the detection of ample carbonates dispersed within the subsurface, thereby strengthening arguments that CO₂ played a major role in the early martian climate system.

Carbon’s geochemical cycle, as originally modeled for Earth, involves interactions between the atmosphere and the hydrosphere that can dramatically influence global climate. Atmospheric CO₂ goes into solution in liquid water relatively easily and can stay there in cool conditions. This alone can draw down a large amount of CO₂. Furthermore, the resulting fluid, carbonic acid (H₂CO₃), reacts with the silicate minerals of the crust to weather the rock and release calcium, magnesium, and iron ions into the water. These ions can promote the precipitation of solid carbonates, (Ca,Mg,Fe)CO₃. Because carbonates remain stable under a great variety of near-surface conditions within the crust, they offer an effective and convenient storage medium for sequestering CO₂.

With the evidence for fluvial activity on early (and only early) Mars, progressive “fixing” of atmospheric CO₂ has been consistently favored by climate modelers. This theory adequately explains why, for the first billion years or so, Mars was warmed by an active greenhouse strong enough to support abundant liquid water and an active hydrologic cycle. Given our understanding of the links among atmospheric CO₂ levels, carbon sequestration, and greenhouse warming on Earth, the CO₂ drawdown explanation for martian cli-

The detection of large volumes of carbonates reasserts the importance of carbon dioxide in martian climate history.

mate change seems rational. Unfortunately, this martian climate jigsaw puzzle is missing a key piece: The widespread and/or massive deposits of carbonate minerals are nowhere to be found. Earth-based and orbital spectroscopic studies suggest at best an upper limit of a few percent within observable surface materials, around 20 to 30% of the amount needed for the accepted climate models (4, 5).

The result has been a cottage industry in explanations as to why we don’t see massive carbonate on Mars (6, 7). These explanations have two prominent themes. First is that our base-level assumption of an early martian climate primarily controlled by atmospheric CO₂ is too simplistic, either because other important chemical species were ignored or because the atmosphere simply never had the required mass. Certainly the former is true. Landers, rovers, and orbiters have directly detected an abundance of sulfate-dominated deposits, offering clear evidence that sulfur species played a role in the early martian climate (8). Like CO₂, atmospheric SO₂ is a greenhouse gas whose complex geochemical cycle includes dissolution into surface waters, acid weathering of surface rocks, and precipitation as relatively stable solids (9). CO₂ and SO₂ differ in stability and solubility under changing conditions of pH, pressure, and tem-

Department of Geological Sciences, Case Western Reserve University, Cleveland, OH 44106, USA. E-mail: rph@case.edu

perature, and hence their actions are related in complex and competitive ways. Nonetheless, it is clear that increasing the assumed amount of SO_2 in the early atmosphere decreases both the amount of CO_2 needed for an early martian greenhouse and the amount of carbonate “missing” from surface materials (10). Other active species such as methane and chlorine are known for Mars and must have played roles as well, but our limited ground truth for Mars equally limits our ability to model their effects on its climate.

The second explanation for the missing martian carbonate is that it is hidden, either originally deposited only at depth or covered and interbedded with dust and other sediments. Conveniently, this explanation requires the carbonate to be difficult to see; but given Mars’ history of localized tectonics and aeolian

exhumation, we should occasionally see its feet sticking out from under the bed sheets. Careful study of orbital imagery has revealed fine-scale grabens, “thumbprint” terrains, and other dissected landscapes resembling terrestrial karst terrains, where acidic surface waters dissect laterally extensive carbonate deposits (11). Unfortunately, such landscapes have equally compelling interpretations as periglacial and thermokarst terrains (where the dissolving subsurface deposits are predominantly ice rather than carbonate) (12).

Another way to hide the carbonate is to disperse it widely in the subsurface. In this scenario, carbonate-charged fluids produced by acidic weathering deposit carbonate when temperatures, pH, or water volume change; if such changes are periodic or episodic, the carbonate can be constantly remobilized. The

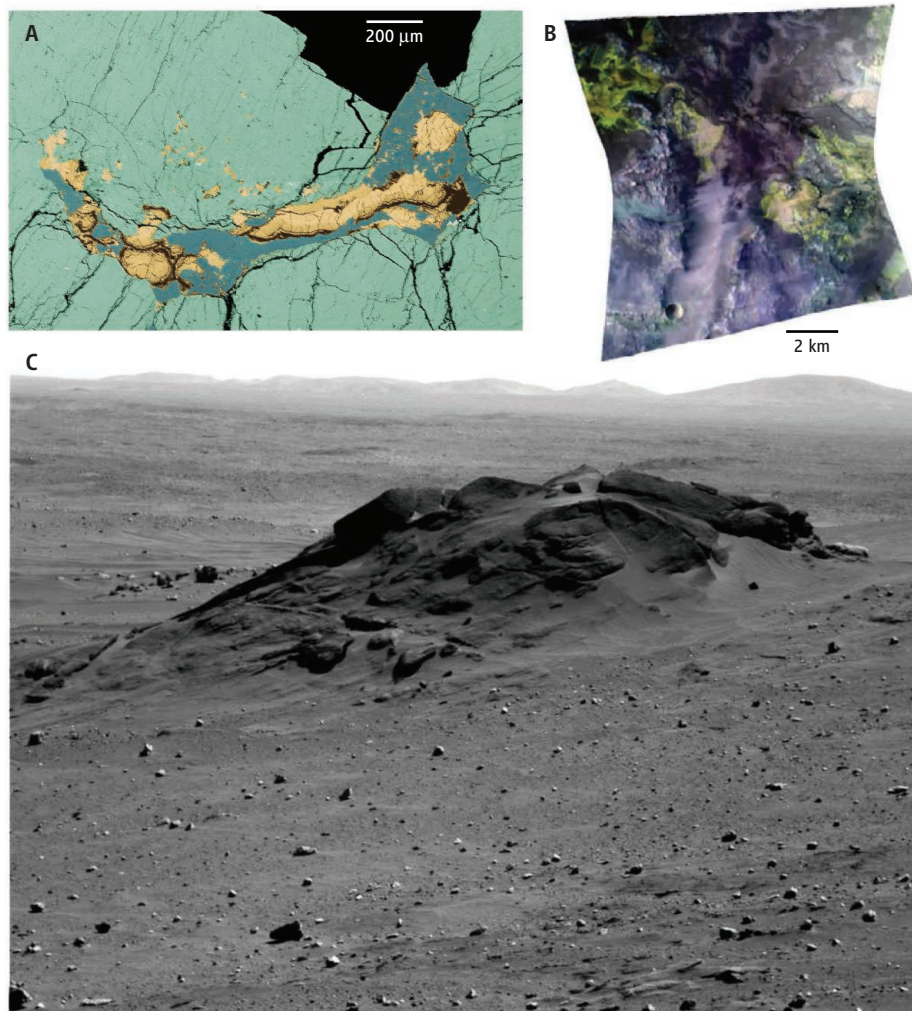
powerful Compact Reconnaissance Imaging Spectrometer for Mars (CRISM) aboard the Mars Reconnaissance Orbiter has enabled the positive identification of carbonate-rich layers in the well-exposed (and well-altered) Nili Fossae area (13) (see the figure, panel B). Also, the Phoenix lander has positively identified carbonate near the martian north polar soils, present at levels high enough to buffer acidic solutions added to samples during onboard analysis (14). There are also samples of distributed martian carbonate: Fractures within the oldest martian meteorite (ALH84001) contain a percent or two of magnesium, iron, and calcium carbonate by volume (15, 16) (see the figure, panel A). Unfortunately, although these observations strongly support distributed carbonate formation on early Mars, they confirm only its presence and do not constrain the amount that may be present.

The findings of Morris *et al.* help carbonate once again take up the leading role in the martian climate change drama. Using nearly every instrument available on the Rover Spirit and 5 years of careful calibration and analysis, the Mars Exploration Rover team has puzzled out the composition of the “Comanche” outcrop on the flanks of the Columbia Hills (see the figure, panel C). The three rocks that make up this outcrop contain roughly 25% (by weight) of carbonate similar in composition to that seen in ALH84001. Although it is possible that the Comanche rocks represent a unique local occurrence rather than an example of something prevalent Mars-wide, the setting (Gusev crater) is far from exceptional, so the latter is certainly plausible. And if such rocks are common, a primordial atmosphere rich in CO_2 levels again makes sense for early Mars.

References

1. M. H. Carr, *Water on Mars* (Oxford Univ. Press, Oxford, 1996).
2. J. B. Pollack, *Icarus* **91**, 173 (1991).
3. R. V. Morris *et al.*, *Science* **329**, 421 (2010); published online 3 June 2010 (10.1126/science.1189667).
4. D. L. Blaney, T. B. McCord, *J. Geophys. Res.* **94**, 10159 (1989).
5. J. L. Bandfield, T. D. Glotch, P. R. Christensen, *Science* **301**, 1084 (2003).
6. A. G. Fairén, D. Fernández-Remolar, J. M. Dohm, V. R. Baker, R. Amils, *Nature* **431**, 423 (2004).
7. D. L. Blaney, *LPI Tech. Rep.* 90-06, 83 (1990).
8. G. Klingelhöfer *et al.*, *Science* **306**, 1740 (2004).
9. N. J. Tosca, S. M. McLennan, *Earth Planet. Sci. Lett.* **241**, 21 (2006).
10. I. Halevy *et al.*, *Science* **318**, 1903 (2007).
11. J. R. Spencer, F. P. Fanale, *J. Geophys. Res.* **95**, 14301 (1990).
12. J. S. Levy, J. W. Head, *Terra Nova* **17**, 503 (2005).
13. B. L. Ehlmann *et al.*, *Science* **322**, 1828 (2008).
14. W. V. Boynton *et al.*, *Science* **325**, 61 (2009).
15. D. W. Mittlefehldt, *Meteoritics* **29**, 214 (1994).
16. C. M. Corrigan, R. P. Harvey, *Meteorit. Planet. Sci.* **39**, 17 (2004).

10.1126/science.1192828



In search of carbonates. Three direct detections of martian carbonate at different scales. (A) False-color backscattered electron image of a thin section of Antarctic martian meteorite ALH84001, sample 303. Fractures within this rock often contain layered or spherical precipitated Mg-Fe-Ca carbonates (orange) with abundances approaching 1% by volume. (B) CRISM false-color composite of CRISM image FRT000093BE showing carbonate-bearing layers (green) detected in the Nili Fossae region of Mars, interbedded with mafic volcanic materials [from (13)]. (C) Left Pancam nonlinearized image of the Comanche outcrop acquired on sol 693 of Spirit's mission to Gusev crater at approximately 14:32:35 Mars local solar time, using filter 7 (432 nm).

ECOLOGY

Filtering Wildlife

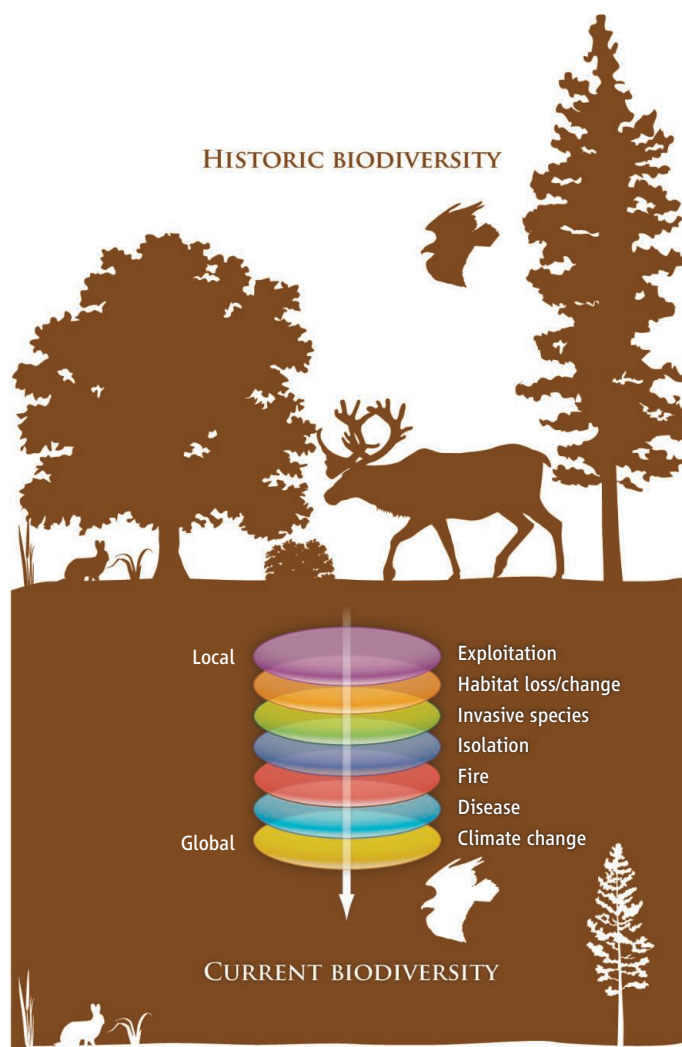
Justin S. Brashares

On 24 April 1903, U.S. President Theodore Roosevelt stood before a crowd outside Gardiner, Montana, and dedicated the world's first national park as a place where "the wild creatures of the Park are scrupulously preserved..." (1). Thirty years later, scientists reported the local extinction of Yellowstone National Park's "white-tailed deer, cougar, lynx, wolf, and possibly wolverine and fisher..." (2, 3). More than 100,000 protected areas worldwide now follow in Yellowstone's footsteps, both in their goal of preserving wildlife and in the difficulties they face in achieving this goal. A growing literature highlights the diverse and complex threats faced by wildlife in protected areas, and the pressing need for better tools and monitoring programs for predicting, understanding, and addressing wildlife declines.

Protected areas are the undisputed backbone of wildlife conservation efforts on land and at sea. Analysts often use the pace and scale of their creation to assess global conservation strategies (4). Yet conservation biologists have long warned that even well-managed protected areas will lose species over time, and of the risks of relying on such areas as reservoirs for nature (5). Recent research suggests that habitat protection reduces extinction rates but also that extinctions occur regularly in protected areas of both developing and developed nations and at rates faster than predicted by conservative models (6, 7). Alarming, there appear to be few obvious patterns to these extinctions that might allow conservationists to predict and protect those species most likely to be at risk (7–10).

At first glance, the causes of wildlife declines in a protected area may seem obvi-

Department of Environmental Science, Policy, and Management, University of California, Berkeley, CA 94720, USA.
E-mail: brashares@berkeley.edu



Filtered out. Wildlife in protected areas (top) face a range of threats that can be envisioned as "extinction filters" (colored disks) passing through an ecological community. Species most vulnerable to a given threat, or to the combined or synergistic impacts of multiple threats, face local extinction (bottom).

ous. Excessive hunting, for instance, effectively extirpated wolves, mountain lions, and other large carnivores from Yellowstone National Park (2). Modern extinctions in protected areas, however, are seldom so easily attributed to one cause. Recent studies link them to an array of threats that vary, often unpredictably, across time and space (7–10). They range from global phenomena such as climate change and atmospheric pollution, to regional issues such as shifts in fire regimes, disease dynamics, or invasive species, to more localized threats such as overharvest, habitat conversion, and the effects of isolation. These threats can interact both additively and synergistically to create syndromes of extinc-

"Extinction filters" help visualize complex threats faced by wildlife in protected areas.

tion that confound diagnosis and remedy (7–11). A final layer of complexity is that each threat can have a unique impact that depends on an animal's vulnerability, as determined by life history and other traits (8).

"Extinction filters" provide a useful, though static, tool for visualizing these complex threats, and predicting wildlife declines in protected areas. In this approach, each threat is envisioned as a filter that passes repeatedly through a wildlife community, selectively removing species most vulnerable to that threat and leaving less susceptible species (12). This framework assumes that (i) each filter selects on different traits of species and at varying strengths over time and space; and (ii) filters may act in conjunction or synergistically to heighten species' vulnerability. Characterizing any threat as a single filter, however, can be misleading. Climate change, for example, often is envisioned as an extinction filter, but realistically may be viewed as a phenomenon that creates or exacerbates myriad other filters (e.g., disease, fire, drought, and habitat change). As with other threats, and shown elegantly in new work by Gilman *et al.* (11) and dis-

cussed elsewhere (8–10), the direct impacts of climate change, fire, disease, and other factors may be dwarfed over time by their indirect effects on species interactions.

In light of this complexity, it is not surprising that even the most informed macro-scale reviews of threats to species and systems fail to identify a "smoking gun" responsible for declines. For example, recent studies of wildlife declines across India (7) and the extinction risk of mammals globally (8, 10) reveal multiple, often overlapping pathways to extinction. Similarly, in a review of declines of marine species and ecosystems, Jackson (9) reasonably devotes equal attention to exploitation, pollution, habitat destruction,

climate change, and the indirect and synergistic impacts of these threats. What might be more surprising is that even micro-level case studies struggle to attribute wildlife declines to a specific cause. Three new studies of wildlife declines in Australia's protected areas demonstrate this point.

In a detailed multiyear study in Kakadu National Park, Woinarski *et al.* (13) systematically surveyed small mammals and observed a 65% decline in species richness and a 75% decline in abundance from 1996 to 2009. Importantly, the researchers suggested that causes of the declines were species-specific and may have involved the individual or combined effects of changes in fire frequency and habitat structure, increases in invasive predators (feral cats and cane toads), and other factors that were not easy to measure. Fire and invasive predators also feature as likely culprits in new research by Firth *et al.* (14) on the extinction of the brush-tailed rabbit-rat in Australia's Garig Gunak Barlu National Park. Here, the authors experimentally determined that dry-season fires significantly reduced wildlife survival, but they also observed population declines in their unburned control areas and concluded that additional threats were at work. In contrast to these studies, Ford *et al.* (15) found that invasive species and fire played no major role in the decline of the brown treecreeper and hooded robin in New South Wales, Australia, but did observe powerful, lagged effects of isolation due to habitat loss and fragmentation over the past 100 years. Together, these studies emphasize the site- and species-specific drivers of wildlife declines. They also emphasize that the factors responsible for endangering a species may be distinct from the challenges experienced by dwindling populations of survivors. This point is illustrated tragically by the recent extinction of mountain caribou in Canada's Banff National Park; years of population decline from habitat loss, isolation, and apparent competition with moose ended with the death of the last known individual in an avalanche (16).

Research over the past 20 years shows that wildlife persistence is often positively associated with the size, connectedness, and remoteness of protected areas and the intactness of surrounding ecosystems (17). Beyond this, though, there are few obvious patterns or golden rules for predicting wildlife declines in protected areas. The lesson from this research, however, is not that we must surrender to the indecipherable complexity of modern declines and resign ourselves to inaction. Instead, we need to move away from broad generalizations and toward

species- and community-specific approaches to conservation. A critical first step is a renewed commitment to wildlife monitoring in protected areas. The nearly exponential growth rate of protected areas since 1903 has greatly outpaced the allocation of resources for monitoring. Governments have used debt relief, foreign aid, direct payments, and other methods to incentivize the creation of protected areas in developing regions. Yet, there exist strikingly few incentives or resources for monitoring the fate of biodiversity in protected areas of the developing or developed world. While conservation science has made headway in quantifying the effectiveness of protected areas, our heavy reliance on habitat cover trends, expert opinion, and questionnaires does not allow us to fully understand or affect the dynamics of wildlife decline. Intensive, long-term monitoring is essential to gaining empirical knowledge of synergies among threats, the role of indirect effects, and other questions critical to minimizing species loss in protected areas.

NEUROSCIENCE

Seeing the Light of Day

Constance Cepko

A potential gene therapy approach could restore some vision to patients with retinitis pigmentosa.

Human beings are highly dependent on vision. It is our most well-developed and cherished sense, giving us color vision with high acuity during the day as well as excellent sensitivity at night. Vision begins with light reception by specialized cells in the retina, a thin sheet of neural tissue that lines the inside of the eyeball (1). Rod photoreceptors provide for sensitivity in dim light, whereas cone photoreceptors provide for color vision in bright light. Unfortunately, photoreceptors are very sensitive to genetic insults. Mutations in more than 200 genes can lead to blindness, more than 40 of which lead to the disease retinitis pigmentosa (2, 3). This disorder typically is due to a mutation in a gene expressed only in rods, and thus individuals with retinitis pigmentosa mutations are often born night-blind. Between ages 20 and 60, cone-mediated vision deteriorates (4), leading to total blindness. The poorly func-

tioning cones in retinitis pigmentosa are the target of a potential gene therapy approach reported by Busskamp *et al.* on page 413 of this issue (5).

When stimulated, most neurons are depolarized and release more neurotransmitter. Photoreceptors are unusual, becoming hyperpolarized when stimulated (by light). Thus, for a signal to mimic light, the signal must hyperpolarize a photoreceptor. Halorhodopsin, a light-activated chloride pump of archaeobacteria (6), does just that. Busskamp *et al.* delivered halorhodopsin [using an adeno-associated vector (AAV)] to cone photoreceptors in two mouse models of retinitis pigmentosa, hoping to bypass the need for the normal light sensor (opsin) in cones and the normal phototransduction process. Indeed, the authors detected light-induced electrical currents in the vector-infected photoreceptor cells, not unlike those measured in normal cones in which light stimulates cone opsin. In a normal retina, neurons that receive signals from photoreceptors extract patterns of interest, such as those that convey the direction of motion

References and Notes

1. L. H. Whittlesey, P. Schullery, *Yellowstone Sci.* **11**, 2 (2003).
2. G. M. Wright, B. H. Thompson, *U.S. Dept. Inter. Wild. Div. Fauna Ser.* **2** (1934).
3. These species are present in Yellowstone National Park today due to reintroduction and recolonization.
4. J. Marton-Lefèvre, *Science* **327**, 1179 (2010).
5. J. M. Diamond, *Biol. Conserv.* **7**, 129 (1975).
6. A. S. L. Rodrigues, *Science* **313**, 1051 (2006).
7. K. K. Karanth *et al.*, *Proc. Biol. Sci.* **277**, 1971 (2010).
8. A. D. Davidson *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 10702 (2009).
9. J. B. C. Jackson, *Proc. Natl. Acad. Sci. U.S.A.* **105**, (suppl. 1), 11458 (2008).
10. J. Schipper *et al.*, *Science* **322**, 225 (2008).
11. S. E. Gilman, M. C. Urban, J. Tewksbury, G. W. Gilchrist, R. D. Holt, *Trends Ecol. Evol.* **25**, 325 (2010).
12. A. Balmford, *Trends Ecol. Evol.* **11**, 193 (1996).
13. J. C. Z. Woinarski *et al.*, *Wildl. Res.* **37**, 116 (2010).
14. R. S. C. Firth, B. W. Brook, J. C. Z. Woinarski, D. A. Fordham, *Biol. Conserv.* **143**, 1193 (2010).
15. H. A. Ford, J. R. Walters, C. B. Cooper, S. J. S. Debus, V. A. J. Doerr, *Biol. Conserv.* **142**, 3182 (2009).
16. M. Hebblewhite *et al.*, *Conserv. Biol.* **24**, 341 (2010).
17. L. R. Prugh, K. E. Hodges, A. R. Sinclair, J. S. Brashares, *Proc. Natl. Acad. Sci. U.S.A.* **105**, 20770 (2008).
18. Funded by the NSF. I thank C. Gurney, C. Golden, T. Bean, C. Burton, K. Fiorella, and L. Prugh for helpful discussion.

10.1126/science.1190095

Department of Genetics and Department of Ophthalmology, Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115, USA. E-mail: cepko@genetics.med.harvard.edu

Sphagnum Moss Disperses Spores with Vortex Rings

Dwight L. Whitaker¹ and Joan Edwards²

Sphagnum moss forms deep mats covering over 1.5 million km² or 1% of Earth's land surface (1) and is important in the global carbon cycle, potentially storing more carbon than any other plant genus (2). With over 285 species (3), often with specific microhabitats, long-distance spore dispersal by wind is critical in maintaining this genus (1). To travel long distances, *Sphagnum* spores must move through the laminar boundary layer into the turbulent boundary layer (up to 10 cm above the ground), where eddies can carry small particles upward and laterally (4). The terminal velocity of *Sphagnum* spores is 5 mm s⁻¹ (5, 6), indicating that any up-draft exceeding this quantity is sufficient to hold them aloft. However, spores with low terminal velocities rapidly decelerate in still air, and dispersal range depends on release height, which is limited for this nonvascular plant. Here, we show that the mechanism *Sphagnum* uses to project spores to heights suitable for dispersal is the generation of turbulent vortex rings: ellipsoidal bubbles of distributed, toroidal vorticity whose in-

ternal flow fields sustain their translational motion (6).

To reach turbulent air, spores are explosively liberated. Our observations show that spores reach heights that cannot be explained by ballistic trajectories. On sunny days capsules dehydrate, and epidermal cells of the capsule body collapse laterally, causing capsules to change shape from spherical to cylindrical (Fig. 1A) and increasing the internal air pressure to between 200 and 500 kPa (7). Eventually pressure forces the cap to break free in under 10⁻⁵ s, vertically propelling the air and spores (Fig. 1D and movies S1 to S4) with an average launch velocity of 16 ± 7 m s⁻¹ (mean ± SEM, *n* = 15, range from 7.9 to 29.8 m s⁻¹) to a mean height of 114 ± 9 mm (mean ± SEM, *n* = 12, range from 90 to 166 mm). These heights cannot be explained by ballistics. Spores launched ballistically with the measured initial velocity of 13 m s⁻¹ would reach a maximum height of only 2 to 7 mm in under 0.5 ms. Whereas after 5 ms, spores traveled over 40 mm while still moving at 3 m s⁻¹ (Fig. 1C).

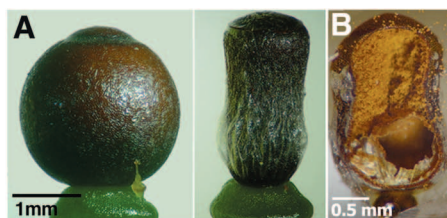
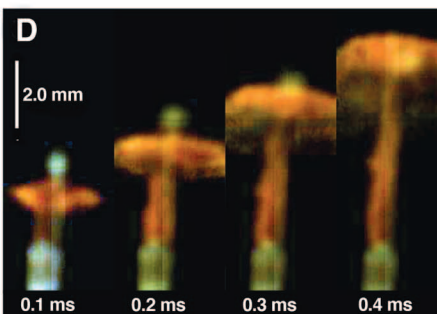
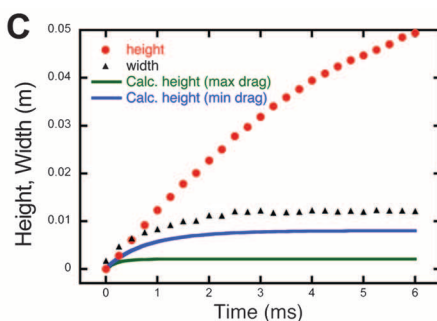


Fig. 1. (A) *Sphagnum affine* capsule transformed from round to cylindrical. (B) A longitudinal section of a capsule showing spore distribution before exploding. (C) Trajectory of spore launch. The observed height (circles) and width (triangles) of the spore cloud versus time from video recorded at 4000 frames per s. Blue and green curves are predicted trajectories of a single spore ballistically launched through still air (6). (D) Sequential video frames of an exploding capsule recorded at 10,000 frames per s with 20-μs exposure.



High-speed videos indicate that the generation of turbulent vortex rings by exploding capsules is responsible for the continued trajectory. The sudden release of air and entrained spores from the circular capsule opening provides the cylindrically symmetric impulse required to generate vortex rings (8). The spores in the images reveal the characteristic mushroom cloud indicative of turbulent vortex rings (Fig. 1D and movies S2 and S3) (6). Spores uniformly distributed over a height of 1 mm in the capsule (Fig. 1B) are concentrated to a height of only 0.3 mm in the vortex bubble that grows to a constant width (Fig. 1C). The drag coefficient, *C_D*, for the vortex bubble in Fig. 1C is between 0.06 and 0.17 (6). This agrees with drag measured for vortex rings in water (*C_D* = 0.09 ± 0.01) (9) and indicates the efficiency with which *Sphagnum* vortex rings travel through still air.

Vortex rings are commonly generated by animals such as jellyfish and squid for propulsion (10). Here, we report vortex rings generated by a plant. The enhanced dispersal from vortex rings explains in part the success of *Sphagnum*, a nonvascular plant that has thrived even after the appearance of land plants with the benefit of well-developed vascular systems.

References and Notes

1. H. Rydin, J. Jeglum, *The Biology of Peatlands* (Oxford Univ. Press, Oxford, 2006).
2. R. S. Clymo, P. M. Hayward, *Bryophyte Ecology*, A. J. E. Smith, Ed. (Chapman and Hall, New York, 1982).
3. C. B. McQueen, R. E. Andrus, in *Flora of North America North of Mexico*, Flora of North America Editorial Committee, Ed. (Oxford Univ. Press, Oxford, 2007), vol. 27, pp. 45–101.
4. P. H. Gregory, *The Microbiology of the Atmosphere* (Wiley, New York, ed. 2, 1973).
5. S. Sundberg, *Ann. Bot. (London)* **105**, 291 (2010).
6. Materials and methods are available as supporting material on Science Online.
7. S. Nawaschin, *Flora* **83**, 151 (1897).
8. G. K. Batchelor, *An Introduction to Fluid Dynamics* (Cambridge Univ. Press, Cambridge, 1967).
9. T. Maxworthy, *J. Fluid Mech.* **64**, 227 (1974).
10. J. O. Dabiri, *Annu. Rev. Fluid Mech.* **41**, 17 (2009).
11. This work was supported in part by NSF Major Research Instrumentation award 0722532. The authors thank D. C. Smith and C. D. Mitescu for comments; R. Andrus for species identification; and E. Chang, A. Conwill, C. Hard, M. Hirai, N. Mitchell, and N. Piatczyc for technical assistance.

Supporting Online Material

www.sciencemag.org/cgi/content/full/329/5990/406/DC1
Materials and Methods

Fig. S1

References

Movies S1 to S4

29 March 2010; accepted 26 May 2010

10.1126/science.1190179

¹Department of Physics and Astronomy, Pomona College, Claremont, CA 91711, USA. E-mail: dwight.whitaker@pomona.edu

²Biology Department, Williams College, Williamstown, MA 01267, USA. E-mail: joan.edwards@williams.edu

Reactivation of Hepatic EPO Synthesis in Mice After PHD Loss

Yoji Andrew Minamishima and William G. Kaelin Jr.*

The hormone erythropoietin (EPO) promotes red blood cell production. The liver is the major source of EPO during embryogenesis. After birth, however, the kidney assumes this role, and hepatic EPO production is largely silenced. This switch has profound medical implications. For example, 20 million Americans have renal disease, of whom 10 to 20% are anemic. Treatment with pharmacological doses of recombinant EPO is costly, requires parenteral administration, and has been linked to cardiovascular side effects and increased mortality (1).

EPO transcription is regulated by HIF (hypoxia-inducible factor). The HIF α subunit is normally prolyl hydroxylated by a PHD (prolyl hydroxylase domain) protein, leading to its polyubiquitination by the von Hippel-Lindau protein (pVHL) and subsequent degradation. Under hypoxic conditions, however, PHD activity decreases and HIF α accumulates. HIF α , bound to HIF β , transcriptionally activates genes such as *EPO*. The canonical HIF family members, HIF1 α and HIF1 β , and most HIF target genes are ubiquitously expressed. In contrast, expression of EPO and HIF2 α , which regulates EPO in both the liver and kidney, is more restricted.

PHD2 is the primary HIF α prolyl hydroxylase and is required for embryonic development (2). Silencing PHD2 in adult mice causes increased renal, but not hepatic, EPO production and polycythemia (overproduction of red blood cells) (3, 4). PHD1 and PHD3 are nonessential genes that fine-tune the HIF response (5).

The PHDs belong to a superfamily of ~70 2-oxoglutarate-dependent dioxygenases (2). These enzymes can be inhibited with druglike molecules, some of which induce hepatic EPO synthesis in anephric mice (6). Conceivably this reflects their ability to inactivate the PHDs. However, inactivation of other 2-oxoglutarate-dependent di-

oxygenases, including enzymes that affect chromatin, might also be involved.

To determine whether PHD loss is sufficient to reactivate hepatic EPO production, we created mice with livers lacking PHD1, PHD2, PHD3, or combinations thereof by using null (*Phd1* and *Phd3*) and floxed (*Phd2*) alleles (5). *Phd2* or, as a control, *Vhl*, was inactivated by using Cre recombinase driven by a hepatocyte-specific transgene (7). Hepatic inactivation of PHD1, PHD2, or PHD3 alone did not increase EPO or hematocrit values (Fig. 1, A and B, and fig. S1). Consistent with previous data (3), hematocrits were modestly increased in mice lacking PHD1 and PHD3 (Fig. 1B and fig. S1). Loss of all three PHDs, however, dramatically increased EPO and hematocrit values (Fig. 1, A and B, and fig. S1). Indeed, these EPO concentrations vastly exceeded those achieved after renal PHD2 inactivation (4, 5, 7).

HIF α concentrations, particularly HIF2 α concentrations, were conspicuously elevated in livers lacking pVHL or all three PHDs, suggesting that exceeding a threshold amount of HIF2 α is sufficient to drive hepatic *Epo* expression (Fig. 1, A and B). The development of polycythemia in mice expressing a stabilized version of HIF2 α in the liver supports this idea (8). Although hepatic *Vhl* inactivation causes benign tumors (7), we have not observed tumors in PHD-defective mice.

The albumin promoter that we used to express Cre becomes active during late embryogenesis, before completion of the switch from the liver to the kidney as the main EPO source, raising the possibility that PHD inactivation can prevent, but not reverse, silencing of the hepatic *Epo* locus. To address this, we inactivated *Phd2* in 4- to 8-week-old *Phd1*^{-/-}; *Phd2*^{flx/flx}; *Phd3*^{-/-} mice by using a ubiquitously expressed Cre-estrogen receptor fusion protein, which was activated by tamoxifen (Fig. 1C) or by tail vein injection of an adenovirus

encoding green fluorescent protein (GFP)-Cre (fig. S1). Both interventions reactivated hepatic EPO synthesis (Fig. 1C and fig. S1). These effects were specific because they were not seen in *Phd2*^{+/+} mice or in mice given an adenovirus encoding GFP alone (fig. S1).

These findings, together with earlier work, suggest that PHD2 inactivation is sufficient to induce near maximal renal EPO production whereas inactivation of all three PHDs is needed to reactivate hepatic EPO production. Thus, drugs that inhibit all three PHDs will probably be required to treat anemia linked to chronic renal failure, whereas drugs that inhibit PHD2 alone might suffice in nephric patients.

Certain features of HIF agonists suggest that they may be safer than pharmacological doses of EPO. For example, HIF activates a suite of genes dedicated to erythropoiesis and might therefore promote red blood cell production at lower circulating EPO concentrations. Moreover, HIF activates genes, such as inducible nitric oxide synthase, that might lower blood pressure and cardiovascular risk. Whether any of these theoretical benefits are real remains to be determined in clinical trials.

References and Notes

1. J. Bohlius et al., *Lancet* **373**, 1532 (2009).
2. W. G. Kaelin Jr., P. J. Ratcliffe, *Mol. Cell* **30**, 393 (2008).
3. K. Takeda et al., *Blood* **111**, 3229 (2008).
4. Y. A. Minamishima et al., *Blood* **111**, 3236 (2008).
5. Y. A. Minamishima et al., *Mol. Cell. Biol.* **29**, 5729 (2009).
6. M. Safran et al., *Proc. Natl. Acad. Sci. U.S.A.* **103**, 105 (2006).
7. V. H. Haase, J. N. Glickman, M. Socolovsky, R. Jaenisch, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 1583 (2001).
8. W. Y. Kim et al., *EMBO J.* **25**, 4650 (2006).
9. W.G.K. owns equity in and is a paid consultant for Fibrogen, Incorporated, which is developing PHD inhibitors. W.G.K. is a coinventor on a patent related to clinical use of PHD inhibitors that was licensed to Fibrogen, Incorporated.

Supporting Online Material

www.sciencemag.org/cgi/content/full/329/5990/407/DC1

Materials and Methods

Fig. S1

References

25 May 2010; accepted 25 June 2010

10.1126/science.1192811

Howard Hughes Medical Institute, Dana-Farber Cancer Institute and Brigham and Women's Hospital, Boston, MA 02115, U.S.A.

*To whom correspondence should be addressed. E-mail: william_kaelin@dfci.harvard.edu

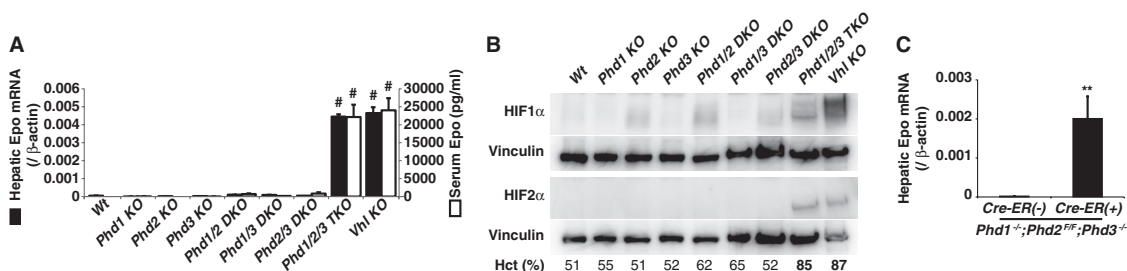


Fig. 1. Hepatic EPO synthesis. (A) Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of hepatic *Epo* mRNA (solid bars) and serum EPO concentrations (open bars) in 4-week-old male mice with indicated genotypes. $n = 3$ mice per group. Pound symbols indicate $P < 0.0001$ comparing indicated genotype versus wild type (wt). DKO, double knockout; TKO, triple knockout. Note

that *Phd2* KO and *Vhl* KO are liver-specific. Error bars indicate 1 SEM. (B) Immunoblots of livers from 4-week-old mice as in (A). Mean hematocrits (Hct) are also shown. (C) Real-time RT-PCR analysis of hepatic *Epo* mRNA from 4-week-old mice with indicated genotypes 1 week after tamoxifen treatment. $n = 3$. $**P < 0.01$. Error bars indicate 1 SEM.

Switched Magnetospheric Regulation of Pulsar Spin-Down

Andrew Lyne,^{1*} George Hobbs,² Michael Kramer,^{1,3} Ingrid Stairs,⁴ Ben Stappers¹

Pulsars are famed for their rotational clocklike stability and their highly repeatable pulse shapes. However, it has long been known that there are unexplained deviations (often termed timing noise) from the rate at which we predict these clocks should run. We show that timing behavior often results from two different spin-down rates. Pulsars switch abruptly between these states, often quasi-periodically, leading to the observed spin-down patterns. We show that for six pulsars the timing noise is correlated with changes in the pulse shape. Many pulsar phenomena, including mode changing, nulling, intermittency, pulse-shape variability, and timing noise, are therefore linked and are caused by changes in the pulsar's magnetosphere. We consider the possibility that high-precision monitoring of pulse profiles could lead to the formation of highly stable pulsar clocks.

Neutron stars form in the supernova collapse of the cores of exhausted massive stars and are composed of some of the densest and most extreme matter in the observable universe. Pulsars are rapidly rotating, highly magnetized neutron stars. As they rotate, intense beams of electromagnetic radiation may sweep across Earth, resulting in pulses that are often observed with radio telescopes, enabling their rotation to be studied with high precision.

Pulsars are among the most stable rotators known in the universe. Over long time spans, the fastest-spinning pulsars, known as millisecond pulsars, even rival the stability of atomic clocks (1). Although they slow down gradually because of the conversion of rotational energy into highly energetic particles and electromagnetic waves, a simple spin-down model using only the pulsar's rotational frequency ν and its first time derivative $\dot{\nu}$ is often sufficient to reveal timing properties that, for instance, allow high-precision tests of the theory of general relativity (2) and may also allow direct detection of gravitational waves (3–5). However, not all pulsars seem to be perfectly stable clocks.

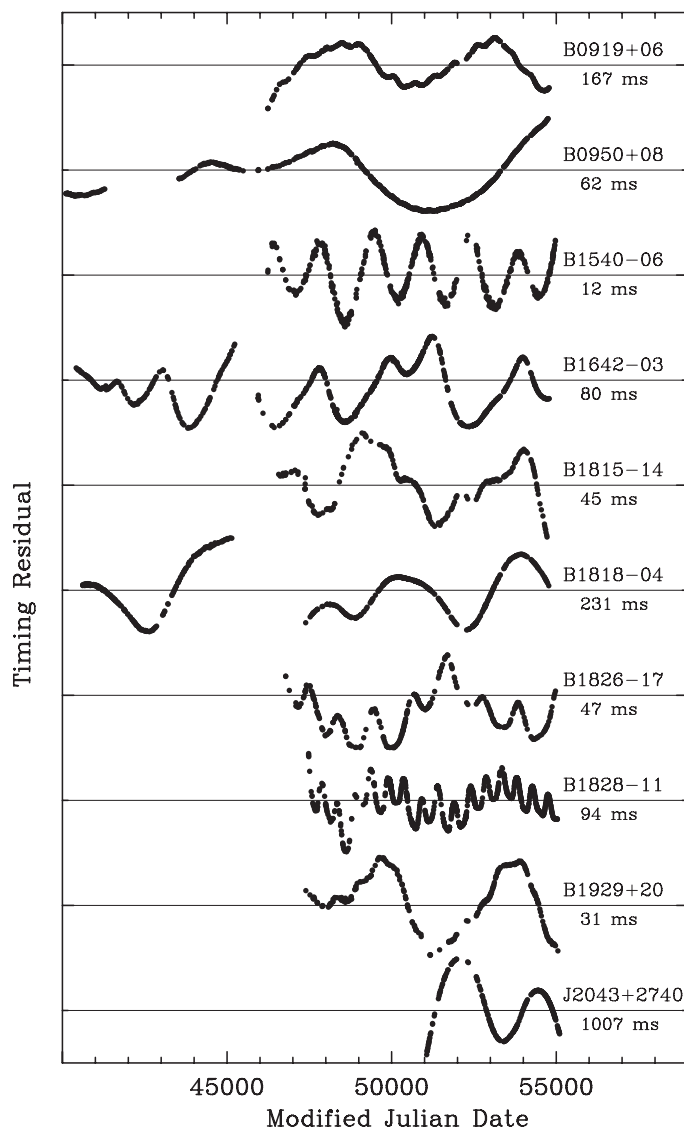
The pulsar timing technique (6, 7) is used to compare pulse arrival times at an observatory with times predicted from a spin-down model. Many pulsars show a phenomenon known as timing noise, in which seemingly quasi-random walks in the rotational parameters are observed. The largest study of such a kind (8) recently presented the rotation properties of 366 pulsars, measured mainly with the Lovell Telescope at Jodrell Bank. This long-term monitoring of pulsars over 40 years made it possible to study phenomena in many pulsars over

decadal time scales. It was shown that the majority of the pulsars were found to have significant irregularities in their rotation rate. The differences be-

tween the observed and predicted times, known as the pulsar timing residuals, can be less than a few milliseconds over more than 30 years, but in other cases timing residuals can be as large as many seconds. In contrast to the standard models held for the past ~40 years, it was found that these timing irregularities are often quasi-periodic, with long (~1- to 10-year) time scales. Here we present a description of these irregularities and how they are related to changes in pulse shape, linking many peculiar and unexplained time-dependent phenomena observed in pulsars.

Pulsar time scales. Many of the properties of pulsars are not perfectly stable, and they vary over a wide range of time scales. Rotational periods range from milliseconds to seconds. The structure and brightness of individual pulses are observed to vary significantly, but the average of many hundreds of individual pulses (on a time scale of minutes) is usually stable, leading to a characteristic profile that is often unique to a pulsar. On time scales of seconds to hours, some pulsars are observed to exhibit

Fig. 1. Pulsar timing residuals relative to a simple spin-down model of the pulse frequency and its first derivative. For PSRs B0919–06, B1540–06, and B1828–11, we have also included the frequency second derivative in the model. For each pulsar, the peak-to-peak range in residual is given, and the vertical scale has been adjusted to give the same peak-to-peak deflection in the diagram. We used data updated from those presented in (8) and also included data for PSR J2043+2740. The residuals were obtained with the TEMPO2 software package (33).



¹Jodrell Bank Centre for Astrophysics, School of Physics and Astronomy, University of Manchester, Manchester M13 9PL, UK. ²Australia Telescope National Facility, CSIRO, Post Office Box 76, Epping, New South Wales 1710, Australia. ³MPI für Radioastronomie, Auf dem Hügel 69, 53121 Bonn, Germany. ⁴Department of Physics and Astronomy, University of British Columbia, 6224 Agricultural Road, Vancouver, BC V6T 1Z1, Canada.

*To whom correspondence should be addressed. E-mail: andrew.lyne@manchester.ac.uk

either nulling events, during which the pulse emission switches off, or mode-changing events in which the observed pulse profile changes abruptly between two (sometimes three) well-defined shapes. On longer time scales, PSR B1931+24 has recently been described as an intermittent pulsar, which relates to the fact that it undergoes extreme nulling events (9), displaying quasi-periodic behavior in which the pulsar acts as a normal pulsar for typically 5 to 10 days before switching off, being undetectable for ~ 25 days, and then abruptly switching on again. On even longer time scales, stable harmonically related periodicities of ~ 250 , 500, and 1000 days have been reported in the rotation rate and pulse shape of PSR B1828–11. The periodicities have generally been interpreted as being caused by PSR B1828–11 freely precessing (10), even though it had been argued that this was not possible in the presence of the superfluid component believed to exist inside neutron stars (11).

Discrete pulsar spin-down states. The patterns observed in the timing residuals of a sample of 10 pulsars (Fig. 1) are typical of the sample presented in (8) and highlight the main results of that paper: (i) the residuals are dominated by quasi-periodic structures and (ii) the residuals are

generally asymmetric, in that the radii of curvature of local maxima are often consistently different from those of local minima. Clear examples are seen in PSRs B0950+08, B1642–03, B1818–04, B1826–17, and B1828–11. In several cases, notably PSRs B0919+06 and B1929+20, relatively rapid oscillations lie on lower-frequency structures.

Structures in the timing residuals have been widely discussed in the literature. Sudden increases in the pulsar's rotation rate are known as glitches and are explained by the sudden unpinning of superfluid vortices in the interior of the neutron star (12). An apparently related phenomenon known as slow glitches has been described (13, 14), characterized by a slow permanent increase in rotation rate but no substantial change in the slow-down rate, and also identified with the interior of the neutron star. The low-frequency structures seen over short data spans were thought to represent either random walks in the pulse frequency and/or its derivatives (15, 16) arising from instabilities within the neutron star superfluid interior, multiple microglitches (17), free precession of the neutron star (10), asteroid belts (18), magnetospheric effects (19), interstellar or interplanetary medium effects (20), or accretion of material onto

the pulsar's surface (21). Timing residuals for the youngest pulsars in (8) are dominated by the recovery from glitch events, sometimes having occurred before the start of observing. In general, for the remaining pulsars it was shown that, with long data spans, the low-frequency structures are no longer dominant but are now understood as restricted pieces of much longer-term oscillatory structures, often with asymmetric maxima and minima. Any model explaining timing noise therefore needs to explain these commonly occurring features.

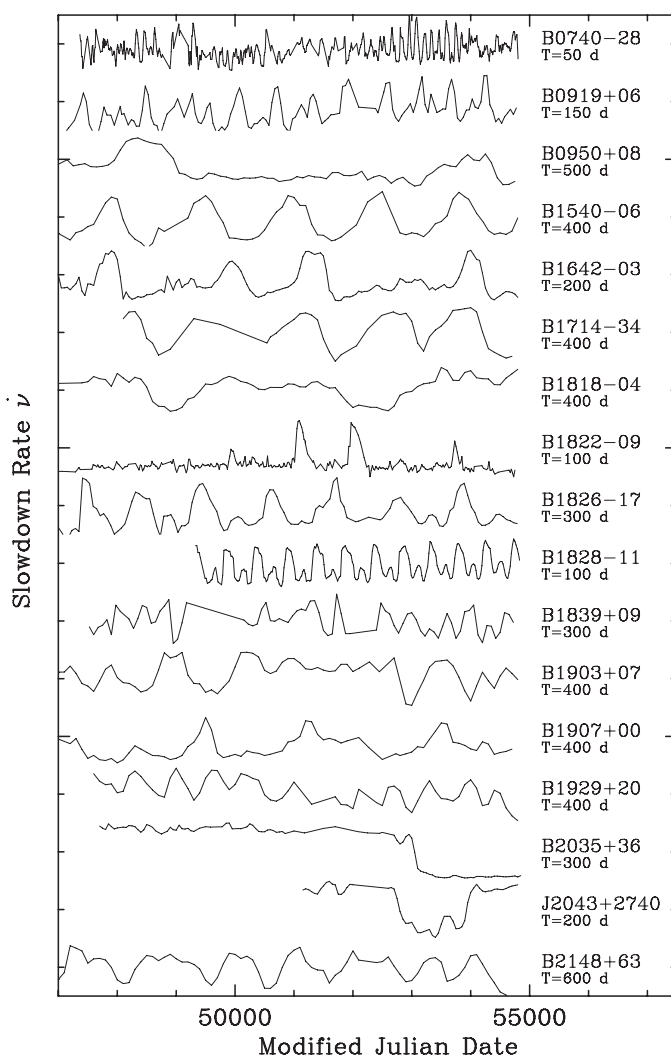
The analysis of PSR B1931+24 (9) showed that the pulsar spin-down rate switched by $\sim 50\%$ between the on and off states, with the pulsar spinning down faster when the radio signal was detectable. The quasi-periodic nature of the time between state changes and the difference in time spent in each state lead naturally to oscillatory, asymmetric timing residuals (fig. S1). The existence of two discrete spin-down rates in PSR B1931+24 and the similarities between such timing residuals and those shown in Fig. 1 suggest that a similar model could be applied to the timing noise seen in all pulsars.

The variation in the spin-down rate $\dot{\nu}(t)$ for 17 pulsars demonstrates that the observed structures in the timing residuals arise from a pulsar's $\dot{\nu}$ moving between a small number of values, and frequently in an oscillatory manner (Fig. 2). In some cases, more complex structure is seen. For instance, in PSR B1642–03, we observe peaks in $\dot{\nu}$ followed by a sudden change to a more negative $\dot{\nu}$ value before a slow gradual rise. In PSR B1828–11, in addition to the oscillatory structure, we observe a long-term gradual linear change in $\dot{\nu}$ across the data span. We concentrate on the dominant features of this figure: The value of $\dot{\nu}$ changes between a few (typically two) well-defined values, often in a quasi-periodic manner.

In order to quantify the behavior, we measured the peak-to-peak values of $\dot{\nu}$ for each pulsar (Table 1). Additionally, for each of the time sequences in Fig. 2, we have performed Lomb-Scargle (22) and wavelet (23) spectral analyses. As expected, some of the resultant spectra (figs. S2 and S3) show narrow, highly periodic features, whereas others show broader, less well-defined peaks.

Pulse-shape variations. Following the implications of the study of PSR B1931+24 that changes within the magnetosphere are responsible for variations in both the spin-down rate and the emission process (9), we have sought changes in the pulse shapes of pulsars that showed the greatest fractional changes in spin-down rate in the timing noise study. Six pulsars show changes in pulse shape that are clearly visible (Fig. 3). From inspection of the profiles in Fig. 3, for each pulsar we selected the simplest shape parameter that would discriminate between the two extreme pulse-shape states, such as the full widths at 10, 50, or 75% (W_{10} , W_{50} , or W_{75}) or W_{eq} , the equivalent width (see the supporting online material for details on how these were determined and their implications for the timing residuals). For six pulsars, the observed changes in $\dot{\nu}$ are indeed directly related to changes in pulse shape (Fig. 4). In most cases, the two quantities clearly track one another,

Fig. 2. Variations in the spin-down rate $\dot{\nu}$ for 17 pulsars during the past 20 years. We determined these values by selecting small sections of data of length T and fitting for values of ν and $\dot{\nu}$, repeating at intervals of $\sim T/4$ through each data set. The chosen value of T is the smallest required to provide sufficient precision in $\dot{\nu}$ and is given below each pulsar name. T is typically 100 to 400 days, so that any short-time-scale variations will be smoothed out. For each pulsar, we adjusted the vertical scale to give the same peak-to-peak amplitude and subtracted an arbitrary vertical offset. Because $\dot{\nu}$ is negative, an increase in the rate of spin-down is represented by a downward deflection in this diagram.



and there is strong evidence for either correlation or anticorrelation in all six cases (fig. S4). It is not clear whether the imperfect correlations are intrinsic or arise from the sparse sampling of the time series or from measurement errors.

Some of the pulse profiles suggest that increased $|\dot{\nu}|$ is associated with increased amplitude of the central (often described as core) emission relative to the surrounding (or conal) emission (Fig. 3). PSR B1822–09 exhibits a main pulse, a precursor, and an interpulse (24, 25). For the high- $|\dot{\nu}|$ state, the precursor is weak and the interpulse is strong, with the reverse occurring for the smaller- $|\dot{\nu}|$ state. Clearly, some changes in $\dot{\nu}$ are associated with large profile changes (for example, PSRs J2043+2740 and B1822–09), whereas smaller profile changes are also observable if sufficiently high-quality data are available (as in PSR B1540–06).

Although the main impression given by the traces in Fig. 4 is that they are bounded by two extreme levels, there are substantial, and often repeated, subtle changes that are synchronized in both shape parameter and $\dot{\nu}$. The shape parameters for the observations of PSRs B1822–09, B1828–11, and B2035+36 imply that they spend most of the time in just one extreme state or the other. This is essentially the phenomenon of mode changing, which has been known since shortly after the discovery of pulsars (26–28, 24). In those papers, pulsars are reported to show stable profiles but suddenly switch to another stable mode for times ranging from minutes to hours. However, the time-averaged values of the shape parameters depend on the mix of the two states over the averaging period, and that varies with time, causing the slower changes in the shape-parameter curves and the spin-down rate curves. About 2500 days of detail in the shape parameters and spin-down rates of PSRs B1822–09 and B1828–11 (Fig. 5) illustrate how a slowly changing mix of the two states is reflected in the form of the smoothed shape curves. In PSR B1822–09, the events centered on modified Julian dates 51100 and 52050 are the sites of slow glitches (13, 14), which we confirm are not a unique phenomenon (8) but arise from short periods of time spent predominantly in a small- $|\dot{\nu}|$, large-precursor mode.

Discussion. The large number of pulsars observed over many years in the Jodrell Bank data archive has allowed the identification of a substantial number of pulsars that have large changes in $\dot{\nu}$, some of which also have detectable, correlated pulse-shape changes. This correlation indicates that the causes of these phenomena are linked and are magnetospheric in origin. The physical mechanism for this link is likely to be that suggested to explain the relationship between spin-down rate and radio emission in B1931+24; namely, a change in magnetospheric particle current flow (9). An enhanced flow of charged particles causes an increase in the braking torque on the neutron star and also in the emission radio waves.

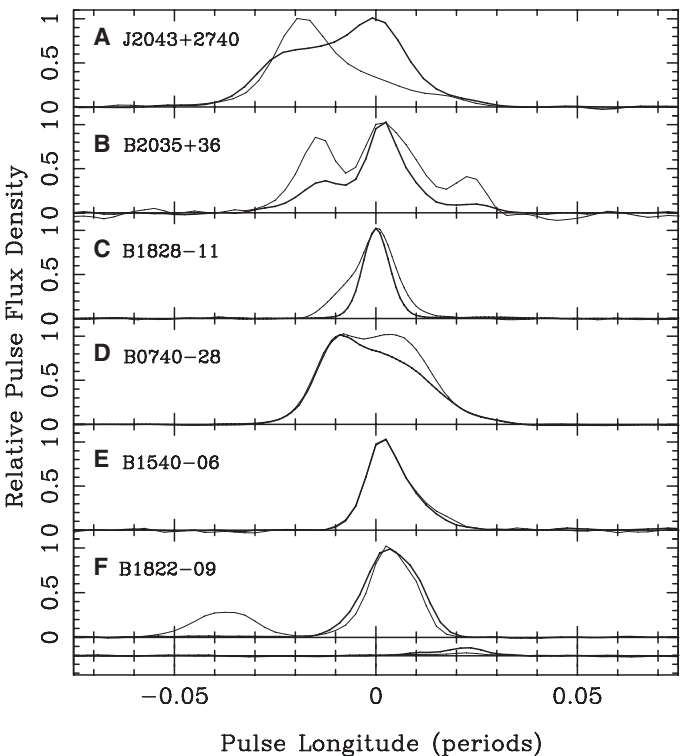
The link between the spin-down rate and radio emission properties has not been established previously, mainly because the time scales of the long-established phenomena of mode changing and

Table 1. Measured parameters of 17 pulsars presented in Fig. 2, as well as PSR B1931+24, which is also discussed in the text. We give the pulsar names, rotational frequency ν , and the first derivative $\dot{\nu}$, followed by the peak-to-peak fractional amplitude $\Delta\nu/\nu$ of the variation seen in Fig. 2. The pulsars are given in order of decreasing value of this quantity. We also present the fluctuation frequencies F of the peaks of the Lomb-Scargle power spectra (fig. S2), with the widths of the peaks or group of peaks given in parenthesis in units of the last quoted digit.

Pulsar name	J2000 name	ν (Hz)	$\dot{\nu}$ (Hz s ^{−15})	$\Delta\nu/\nu$ (%)	F (year ^{−1})	Comment
B1931+24*	J1933+2421	1.229	−12.25	44.90	13.1(7)	Intermittent pulsar
B2035+36	J2037+3621	1.616	−12.05	13.28	0.02(2)	28% change in W_{eq}
B1903+07	J1905+0709	1.543	−11.76	6.80	0.36(13)	
J2043+2740	J2043+2740	10.40	−135.36	5.91	0.11(5)	100% change in W_{50}
B1822–09	J1825–0935	1.300	−88.31	3.28	0.40(7)	100% change in $A_{\text{pc}}/A_{\text{mp}}$
B1642–03	J1645–0317	2.579	−11.84	2.53	0.26(7)	
B1839+09	J1841+0912	2.622	−7.50	2.00	1.00(15)	
B1540–06	J1543–0620	1.410	−1.75	1.71	0.24(2)	12% change in W_{10}
B2148+63	J2149+6329	2.631	−1.18	1.69	0.33(7)	
B1818–04	J1820–0427	1.672	−17.70	0.85	0.11(1)	
B0950+08	J0953+0755	3.952	−3.59	0.84	0.07(3)	
B1714–34	J1717–3425	1.524	−22.75	0.79	0.26(4)	
B1907+00	J1909+0007	0.983	−5.33	0.75	0.15(2)	
B1828–11	J1830–1059	2.469	−365.68	0.71	0.73(2)†	100% change in W_{10}
B1826–17	J1829–1751	3.256	−58.85	0.68	0.33(2)	
B0919+06	J0922+0638	2.322	−73.96	0.68	0.62(4)	
B0740–28	J0742–2822	5.996	−604.36	0.66	2.70(20)	20% change in W_{75}
B1929+20	J1932+2020	3.728	−58.64	0.31	0.59(2)	

*Data from reference (9). †Note the presence of a second harmonic at $F = 1.47(2) \text{ year}^{-1}$, seen in fig. S2 and discussed in (10).

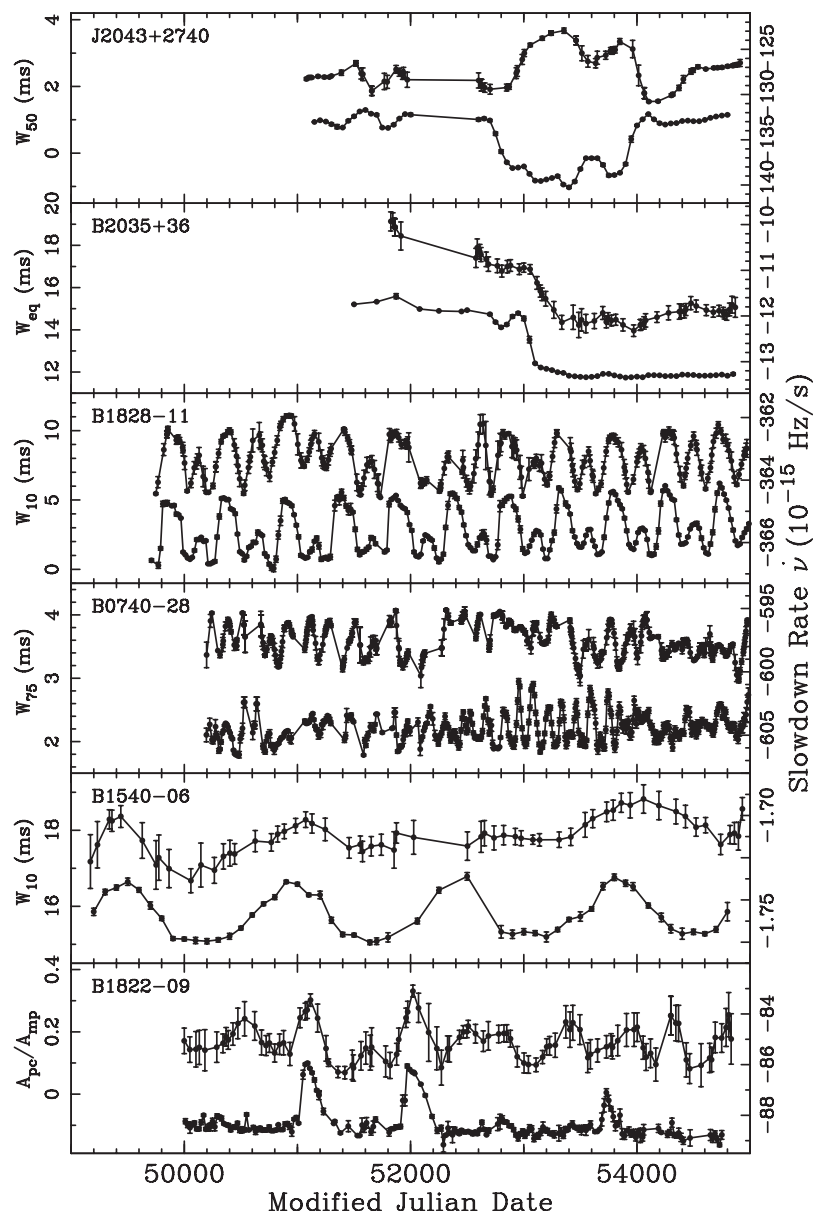
Fig. 3. (A to F) The integrated profiles at 1400 MHz of six pulsars that show long-term pulse-shape changes. For each pulsar, the two traces represent examples of the most extreme pulse shapes observed. The profile drawn in the thick line corresponds to the largest rate of spin-down $|\dot{\nu}|$. The profiles are scaled so that the peak flux density is approximately the same. PSR B1822–09 has an interpulse which is displayed, shifted by half the pulse period, in the second trace below the main pulse.



pulse nulling were much shorter than the time required to measure any change in $\dot{\nu}$. The extended high-quality monitoring of many pulsars has revealed long-term manifestations of these phenomena and allowed their unambiguous association with the spin-down rates of pulsars, seen as timing noise.

Pulsars can spend long periods of time in one magnetospheric state or another, or in some cases switch rapidly back and forth between states, with the fractions of time spent in the two states often varying with time. It has long been suspected that mode changing and nulling are closely related (29, 30).

Fig. 4. The average value of pulse-shape parameter and spin-down rate $\dot{\nu}$ measured for six pulsars. The lower trace in each panel (right-hand scale) shows the same values of $\dot{\nu}$ given in Fig. 2, whereas the upper trace gives a measure of the pulse shape, with the scale given to the left. W_{10} , W_{50} , and W_{75} are the full widths of the pulse profile at 10, 50, and 75% of the peak pulse amplitude, respectively; W_{eq} is the pulse equivalent width (the ratio of the area under the pulse to the peak pulse amplitude); and A_{pc}/A_{mp} is the ratio of the amplitudes of the precursor and main pulse. The time over which a shape parameter is averaged is the same as the time T given in Fig. 2 for the fitting of $\dot{\nu}$. The uncertainty on a shape parameter is derived from the standard deviation of the individual values used to determine the average.



The intermittent pulsar B1931+24 has the largest fractional change in $\dot{\nu}$ in Table 1 and, as it completely disappears, also has the largest apparent change in pulse shape. Mode changing and nulling therefore probably differ only in the magnitude of the changes in the magnetospheric current flows. There is a close linear relationship between $\Delta\dot{\nu}$ and the spin-down rate $|\dot{\nu}|$ (fig. S5), indicating that the value of $\dot{\nu}$ switches by about 1% of the mean value, independent of its magnitude.

We must also emphasize that (i) the fast change between the states indicates that the magnetospheric state changes on a fast time scale, but can then be stable for many months or years before undergoing another fast change; (ii) whatever the cause of the state switching, for most pulsars it is not driven by a highly periodic (high-Q) oscillation; and (iii) increased $|\dot{\nu}|$ is associated with increased amplitude of the core emission relative to conal emission. The fast state changes seem to rule

out free precession as the origin of the oscillatory behavior. PSR B1828-11 was considered unique in that it was the only pulsar that showed clear evidence for free precession (10). Our model indicates that this pulsar is not unique and exhibits the same state-changing phenomenon shown here for other pulsars.

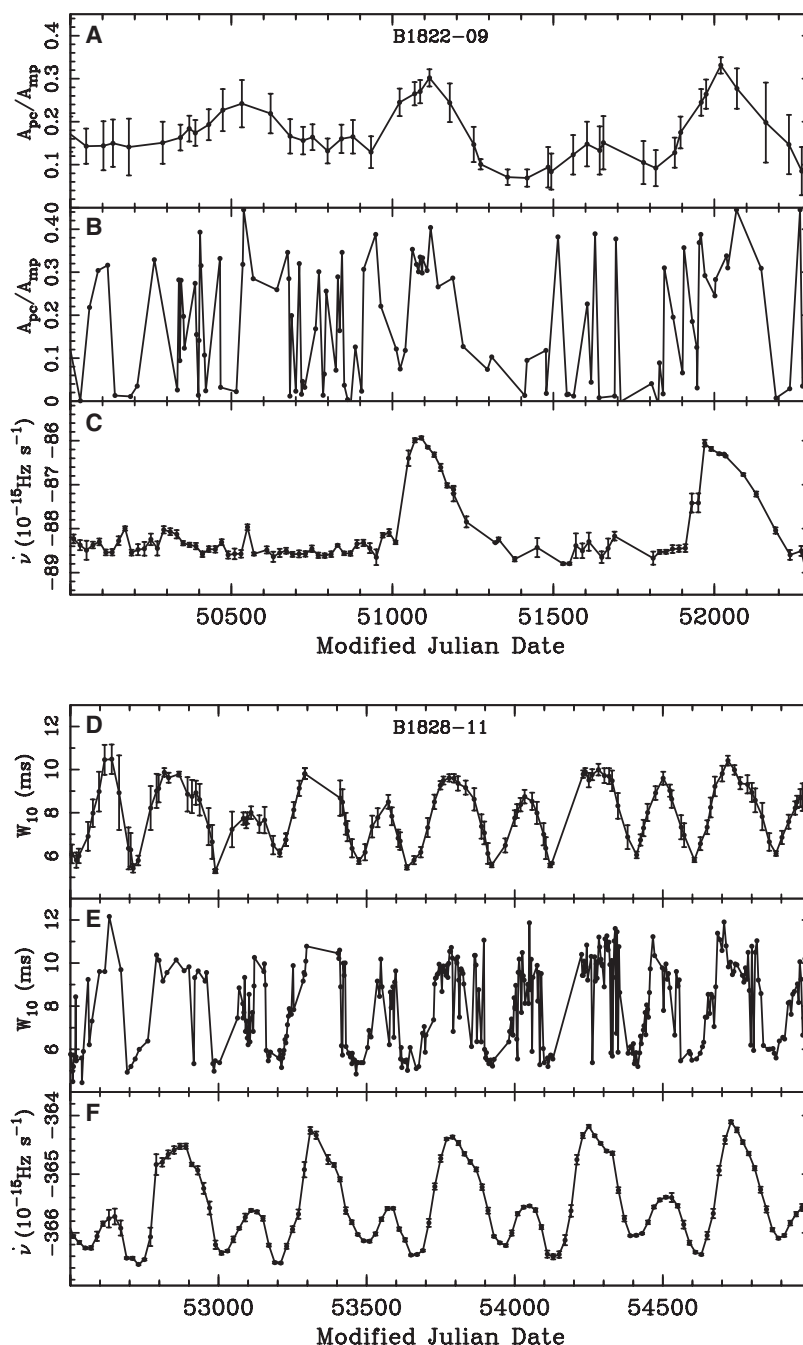
If we could monitor a pulsar continuously, its magnetospheric state at any given time could be determined from the pulse shape. The state gives a measure of the spin-down rate, allowing the timing noise to be removed (an example is given in fig. S1). The most stable millisecond pulsars are being regularly observed from many observatories worldwide in the hope of making the first direct detection of gravitational waves (31). The first-discovered millisecond pulsar, PSR B1937+21, can be timed with high precision (of ~ 100 ns) over short data spans, but low-frequency timing irregularities dominate the timing residuals over data spanning more

than ~ 3 years (32), making this pulsar potentially unusable for gravitational wave detection experiments. However, if magnetospheric state switching is responsible and can be applied to millisecond pulsars, then the timing irregularities can be modeled and removed, raising the possibility of producing an essentially stable clock.

References and Notes

1. G. Petit, P. Tavella, *Astron. Astrophys.* **308**, 290 (1996).
2. M. Kramer et al., *Science* **314**, 97 (2006).
3. M. V. Sazhin, *Sov. Astron.* **22**, 36 (1978).
4. S. Detweiler, *Astrophys. J.* **234**, 1100 (1979).
5. F. A. Jenet, G. B. Hobbs, K. J. Lee, R. N. Manchester, *Astrophys. J.* **625**, L123 (2005).
6. D. R. Lorimer, M. Kramer, *Handbook of Pulsar Astronomy* (Cambridge Univ. Press, Cambridge, 2005).
7. R. T. Edwards, G. B. Hobbs, R. N. Manchester, *Mon. Not. R. Astron. Soc.* **372**, 1549 (2006).
8. G. Hobbs, A. G. Lyne, M. Kramer, *Mon. Not. R. Astron. Soc.* **402**, 1027 (2010).
9. M. Kramer, A. G. Lyne, J. T. O'Brien, C. A. Jordan, D. R. Lorimer, *Science* **312**, 549 (2006).

Fig. 5. The variations in pulse-shape parameters for PSR B1822–09 (A to C) and PSR B1828–11 (D to F). Traces (A), (C), (D), and (F) are taken from Fig. 4 and show the smoothed values of shape parameter and spin-down rate for the two pulsars, whereas traces (B) and (E) show the values of shape parameter for individual observations, which are typically between 6 and 18 min duration. For both pulsars, individual shape parameter values typically take either a high or low value.



10. I. H. Stairs, A. G. Lyne, S. L. Shemar, *Nature* **406**, 484 (2000).
11. J. Shaham, *Astrophys. J.* **214**, 251 (1977).
12. P. W. Anderson, N. Itoh, *Nature* **256**, 25 (1975).
13. W. Z. Zou *et al.*, *Mon. Not. R. Astron. Soc.* **354**, 811 (2004).
14. T. V. Shabanova, *Astrophys. Space Sci.* **308**, 591 (2007).
15. P. E. Boynton *et al.*, *Astrophys. J.* **175**, 217 (1972).
16. J. M. Cordes, G. S. Downs, *Astrophys. J. Suppl. Ser.* **59**, 343 (1985).
17. G. H. Janssen, B. W. Stappers, *Astron. Astrophys.* **457**, 611 (2006).
18. J. M. Cordes, R. M. Shannon, *Astrophys. J.* **682**, 1152 (2008).
19. K. S. Cheng, *Astrophys. J.* **321**, 799 (1987).
20. K. Scherer, H. Fichtner, J. D. Anderson, E. L. Lau, *Science* **278**, 1919 (1997).
21. G. J. Qiao, Y. Q. Xue, R. X. Xu, H. G. Wang, B. W. Xiao, *Astron. Astrophys.* **407**, L25 (2003).
22. J. D. Scargle, *Astrophys. J.* **263**, 835 (1982).
23. G. Foster, *Astron. J.* **112**, 1709 (1996).
24. L. A. Fowler, D. Morris, G. A. E. Wright, *Astron. Astrophys.* **93**, 54 (1981).
25. D. Morris, D. A. Graham, N. Bartel, *Mon. Not. R. Astron. Soc.* **194**, 7P (1981).
26. D. C. Backer, *Nature* **228**, 1297 (1970).
27. A. G. Lyne, *Mon. Not. R. Astron. Soc.* **153**, 27P (1971).
28. D. Morris, W. Sieber, D. C. Ferguson, N. Bartel, *Astron. Astrophys.* **84**, 260 (1980).
29. A. G. Lyne, F. G. Smith, *Pulsar Astronomy* (Cambridge Univ. Press, Cambridge, ed. 3, 2005).
30. N. Wang, R. N. Manchester, S. Johnston, *Mon. Not. R. Astron. Soc.* **377**, 1383 (2007).
31. G. Hobbs, in *AIP Conference Series*, Y.-F. Yuan, X.-D. Li, D. Lai, Eds. (American Institute of Physics, New York, 2008), vol. 968, pp. 173–180.
32. V. M. Kaspi, J. H. Taylor, M. Ryba, *Astrophys. J.* **428**, 713 (1994).
33. G. B. Hobbs, R. T. Edwards, R. N. Manchester, *Mon. Not. R. Astron. Soc.* **369**, 655 (2006).
34. Pulsar research at the Jodrell Bank Centre for Astrophysics is supported by a Rolling Grant from the UK Science and Technology Facilities Council. G.H. is the recipient of an Australian Research Council QEII Fellowship (no. DP0878388). M.K. is supported by a salary from the Max-Planck Society. Pulsar research at the University of British Columbia is supported by a Natural Sciences and Engineering Research Council Discovery Grant.

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1186683/DC1
SOM Text
Figs. S1 to S5
References

5 January 2010; accepted 11 June 2010

Published online 24 June 2010;

10.1126/science.1186683

Include this information when citing this paper.

Genetic Reactivation of Cone Photoreceptors Restores Visual Responses in Retinitis Pigmentosa

Volker Busskamp,^{1,2*} Jens Duebel,^{1*} David Balya,^{1*} Mathias Fradot,^{3,4,5} Tim James Viney,¹ Sandra Siegert,¹ Anna C. Groner,^{2,6} Erik Cabuy,¹ Valérie Forster,^{3,4,5} Mathias Seeliger,⁷ Martin Biel,⁸ Peter Humphries,⁹ Michel Paques,^{3,4,5,10,11} Saddek Mohand-Said,^{3,4,5,10} Didier Trono,^{2,6} Karl Deisseroth,¹² José A. Sahel,^{3,4,5,10,11} Serge Picaud,^{3,4,5,11} Botond Roska^{1†}

Retinitis pigmentosa refers to a diverse group of hereditary diseases that lead to incurable blindness, affecting two million people worldwide. As a common pathology, rod photoreceptors die early, whereas light-insensitive, morphologically altered cone photoreceptors persist longer. It is unknown if these cones are accessible for therapeutic intervention. Here, we show that expression of archaeobacterial halorhodopsin in light-insensitive cones can substitute for the native phototransduction cascade and restore light sensitivity in mouse models of retinitis pigmentosa. Resensitized photoreceptors activate all retinal cone pathways, drive sophisticated retinal circuit functions (including directional selectivity), activate cortical circuits, and mediate visually guided behaviors. Using human ex vivo retinas, we show that halorhodopsin can reactivate light-insensitive human photoreceptors. Finally, we identified blind patients with persisting, light-insensitive cones for potential halorhodopsin-based therapy.

Retinitis pigmentosa (1, 2) is the result of diverse mutations in more than 44 genes expressed in rod photoreceptors (3), which then degenerate, causing loss of night vision. Subsequently, cone photoreceptors, which are responsible for color and high-acuity daytime vision, progressively lose their photoreceptive outer segments, leading to overall blindness. Despite this loss of sensitivity, cone cell bodies remain present longer than rods in both humans and animals (4–6), but it is not known whether these light-insensitive cells can be reactivated or if information from them can still flow to downstream visual circuits (Fig. 1A) for a substantial time window after the loss of photosensitivity (7).

To restore light-evoked activity in light-insensitive cone photoreceptors, we genetically targeted a light-activated chloride pump (8–10), enhanced *Natronomonas pharaonis* halorhodopsin (eNpHR) (11, 12), to photoreceptors by means of adeno-associated viruses (AAVs) (13, 14).

Light-activated chloride pumps are rational candidates for reactivating vertebrate photoreceptors, as both eNpHR-expressing cells (12) and healthy photoreceptors hyperpolarize in response to increases in light intensity. We selected two animal models of retinitis pigmentosa for gene therapy, both of which lead to retinal degeneration (RD). *Cnga3*^{−/−}; *Rho*^{−/−} double-knockout mice served as a model of slow forms of RD (s-RD mice) (15), and *Pde6b*^{rd1} (also known as rd1) mice modeled fast forms of RD (f-RD mice) (16). Targeted expression of eNpHR was accomplished with the use of three cell-specific promoters (Fig. 1B): (i) human rhodopsin (hRHO) (17), (ii) human red opsin (hRO) (18), and (iii) mouse cone arrestin-3 (mCAR) (19).

To assess the effectiveness and specificity of the promoters, we used eNpHR fused to enhanced yellow fluorescent protein (EYFP) (Fig. 2, A to I) (11). It is important to restrict the expression of eNpHR to photoreceptors only, because eNpHR

in downstream retinal circuit elements, such as ON-bipolar and ON-ganglion cells, may inhibit the flow of information across the retina. Enhanced green fluorescent protein (EGFP)-expressing AAVs were used as controls throughout this study (fig. S1). We selected the hRO and mCAR promoters for s-RD mice (Fig. 2, B and C) and the mCAR promoter for f-RD mice (Fig. 2F), on the basis of their ability to selectively drive expression of eNpHR-EYFP in a high percentage of cone photoreceptors (figs. S2 and S3).

The life span of cones in RD mice defines the window of opportunity for reactivation. Therefore, we tested EYFP or EGFP expression driven by the mCAR promoter at different time points during retinal degeneration. Surprisingly, EYFP and EGFP expression lasted more than eight months (Fig. 3A, fig. S4, last tested f-RD at postnatal day 264 (P264) and s-RD at P255), longer than opsin protein expression [~P95 (5) or between P21 and P110 (20)]. We isolated these long-lasting AAV-transduced cells from f-RD retinas at different time points and verified their identity by analyzing their transcriptome (P110 to P220, fig. S5). Cone-specific genes were expressed in the isolated cells, whereas markers of other retinal cell types were absent, suggesting that AAV-labeled cells are altered cones, even at later stages of degeneration. The fact that opsin mRNA remained, whereas the opsin protein did not, suggests translational down-regulation of opsins, as also shown before (20). We estimate that ~27% of cones remain between P184 and P255 in s-RD and ~25% remain between P182 and P264 in f-RD mice (Fig. 3A).

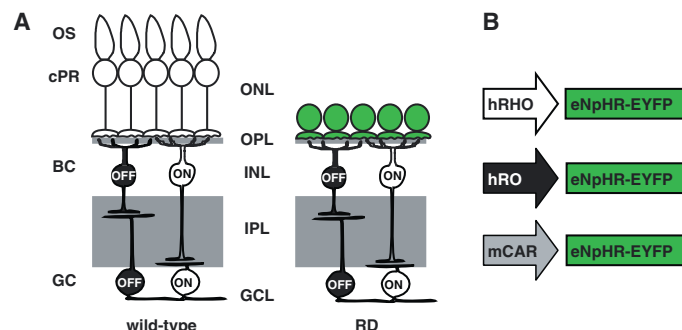
We tested whether eNpHR drives cone light responses in RD retinas at an age (P53 to P264, table S1) when many (s-RD) (15) or all (f-RD) (20) rods have already died. eNpHR-EYFP-expressing photoreceptors in s-RD and f-RD retinas displayed larger (Fig. 3B and fig. S6), more sustained (Fig. 3C), and significantly faster (Fig. 3D) photocurrents than those of wild-type (WT) cones. The photocurrents peaked at 580 nm. Photoreceptors expressing only EGFP did not

¹Neural Circuit Laboratories, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland. ²National Centre of Competence in Research Frontiers in Genetics Program, Geneva 1211, Switzerland. ³Inserm, UMR_S968, Institut de la Vision, Paris, France. ⁴Université Pierre et Marie Curie Paris 06, UMR_S968, Institut de la Vision, Paris F-75012, France. ⁵CNRS, UMR_7210, Paris F-75012, France. ⁶School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland. ⁷Division of Ocular Neurodegeneration, Institute for Ophthalmic Research, Department of Ophthalmology II, Eberhard-Karls University, Tübingen, Germany. ⁸Center for Integrated Protein Science Munich and Department of Pharmacy, Ludwig-Maximilians-Universität München, Munich, Germany. ⁹Smurfit Institute of Genetics, Trinity College, Dublin, Ireland. ¹⁰Centre d'Investigation Clinique 503, Inserm-Centre Hospitalier National d'Ophthalmologie des Quinze-Vingts, Paris, France. ¹¹Fondation Ophthalmologie Adolphe de Rothschild, Paris, France. ¹²Department of Bioengineering and Department of Psychiatry and Behavioral Sciences, Stanford University, Stanford, CA 94305, USA.

*These authors contributed equally to this work.

†To whom correspondence should be addressed. E-mail: botond.roska@fmi.ch

Fig. 1. Scheme of the excitatory pathways of cone retinal circuitry in WT and RD retinas. (A) Cone photoreceptors (cPR) in WT retinas (left) detect light with photopigments in their outer segments (OS). Cone cell bodies are located in the outer nuclear layer (ONL). Cones provide input to ON and OFF bipolar cells (BC) that have cell bodies in the inner nuclear layer (INL). Bipolar cells are connected to corresponding ON and OFF ganglion cells (GC). Ganglion cell bodies are in the ganglion cell layer (GCL), and their axons relay visual information to higher visual centers. The locations of inhibitory interactions mediated by horizontal cells in the outer plexiform layer (OPL) and by amacrine cells in the IPL are indicated by gray boxes. Light-insensitive RD cones (right) lack outer segments, but their cell bodies (green) persist longer than rods. (B) AAV-expression constructs. hRHO, hRO, or mCAR promoters drive eNpHR-EYFP.



react to light. The magnitude of photocurrents in eNpHR-transduced s-RD and f-RD mice were similar (Fig. 3B). The current size was independent of the holding voltage (Fig. 3E), a finding that is consistent with the view that the photocurrents

are mediated by ion pumps (10). Photocurrents and voltages were modulated across three logarithmic units of intensities (fig. S6). In the absence of functional outer segments, which normally generate currents that depolarize photoreceptors

in the dark, RD photoreceptors were expected to stay hyperpolarized. A hyperpolarized state would limit the ability of eNpHR currents to modulate synaptic transmission. However, the recorded eNpHR-expressing RD photoreceptors were depo-

Fig. 2. Targeted expression of a light-sensitive chloride pump in persisting photoreceptors in RD retinas. (A to I) Cross sections of GFP-immunostained (35) retinas transduced by hRHO- (A, D, G), hRO- (B, E, H), and mCAR- (C, F, I) eNpHR-EYFP AAVs in s-RD (A to C), f-RD (D to F), and WT (G to I) mice. Left panel of each pair, eNpHR-EYFP; right panel of each pair, co-stained with 4',6'-diamidino-2-phenylindole (DAPI). Scale bar, 20 μ m.

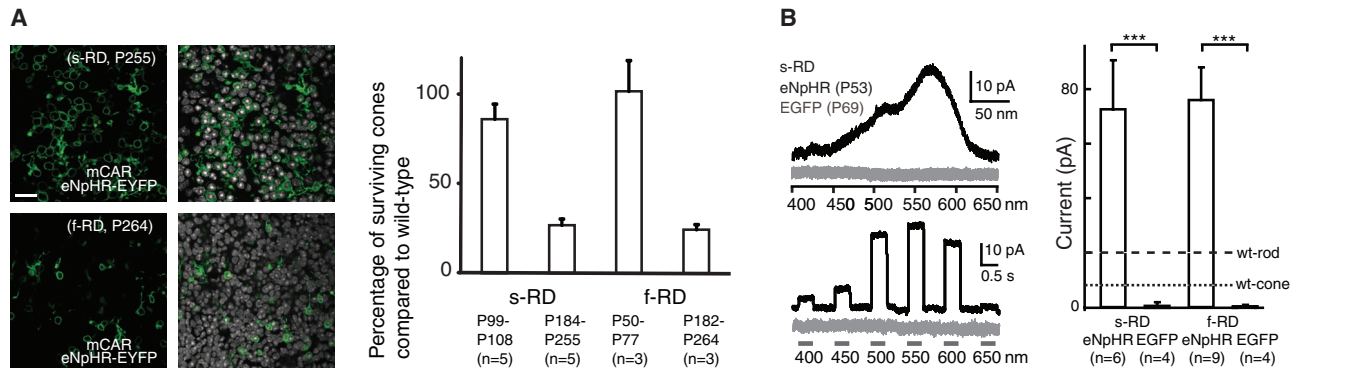
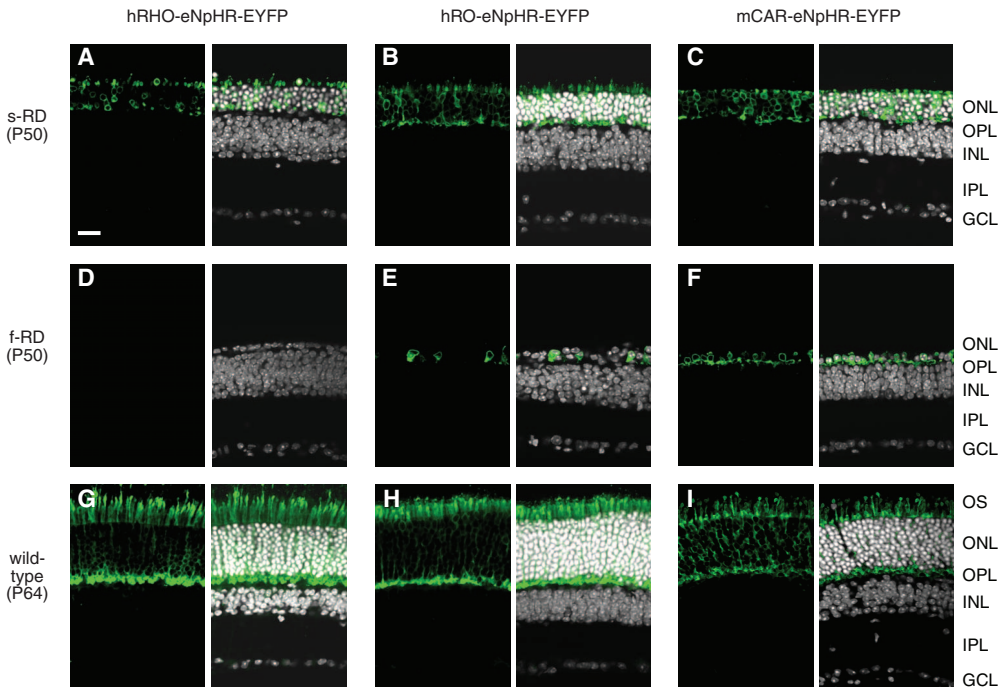


Fig. 3. Light responses in surviving eNpHR-expressing RD cones. (A) (Left) Confocal top views of GFP-immunostained RD photoreceptors transduced by mCAR-eNpHR-EYFP in P255 s-RD (top) and P264 f-RD (bottom) retinas. Left panel of each pair, eNpHR-EYFP; right panel of each pair, co-stained with DAPI. Scale bar 20 μ m. (Right) Estimated fraction of surviving cones (35) in s-RD and f-RD mice at different time points compared with WT cone numbers. n, number of retinas analyzed. (B) (Left) Photocurrent action spectrum of an eNpHR- (black) or EGFP- (gray) expressing s-RD cone. Top, color-ramp; bottom, light flashes. Gray bars indicate the timing of the full-field light stimuli. (Right) Magnitude of photocurrents in photoreceptors expressing eNpHR or EGFP. Dashed lines show the peak magnitude of photocurrents in WT cones (short dashes) and WT rods (long dashes). Stars indicate statistical significance (35). (C) Peak and steady-state photocurrents (0.5-s flash) in RD and

WT animals. (D) Rise and decay time constants of eNpHR-mediated photocurrents compared with the rise time constant in WT photoreceptors. (E) eNpHR-mediated photocurrents in RD retinas at different holding voltages. Because the response properties in s-RD and f-RD mice were similar, the data from both mouse lines were grouped in panels (C to E). All WT cone response data shown in Fig. 3 were taken from (36). Error bars indicate SEM.

larized in the dark [-26 ± 3 mV, $n = 12$ cones; see supporting online material (SOM)].

The ability of eNpHR-reactivated RD photoreceptors to convey information to downstream retinal circuits depends on the presence of functional photoreceptor-to-bipolar cell synapses. As retinal degeneration progresses, these synapses morphologically reorganize (3). However, they still possess elements of both pre- and post-synaptic machinery (5). To test for potential sig-

nal flow from photoreceptors to ganglion cells, we recorded excitatory currents from ganglion cells, the output neurons of the retina. Ganglion cells in eNpHR-transduced s-RD and f-RD retinas displayed robust, light-evoked excitatory currents (Fig. 4A), indicating functional outer and inner retinal synaptic connections. The magnitudes of these excitatory currents were comparable to those in WT retinas (Fig. 4A). There were no measurable light-evoked currents in the

ganglion cells of retinas transfected with EGFP (Fig. 4A).

The retina incorporates two major information channels that diverge at the level of bipolar cells and are physically separated by depth within the inner plexiform layer (IPL). ON cells are activated by light increments, whereas OFF cells are activated by light decrements (21). The activity of ganglion cells is coded by action potentials ("spikes") that form the output signal of

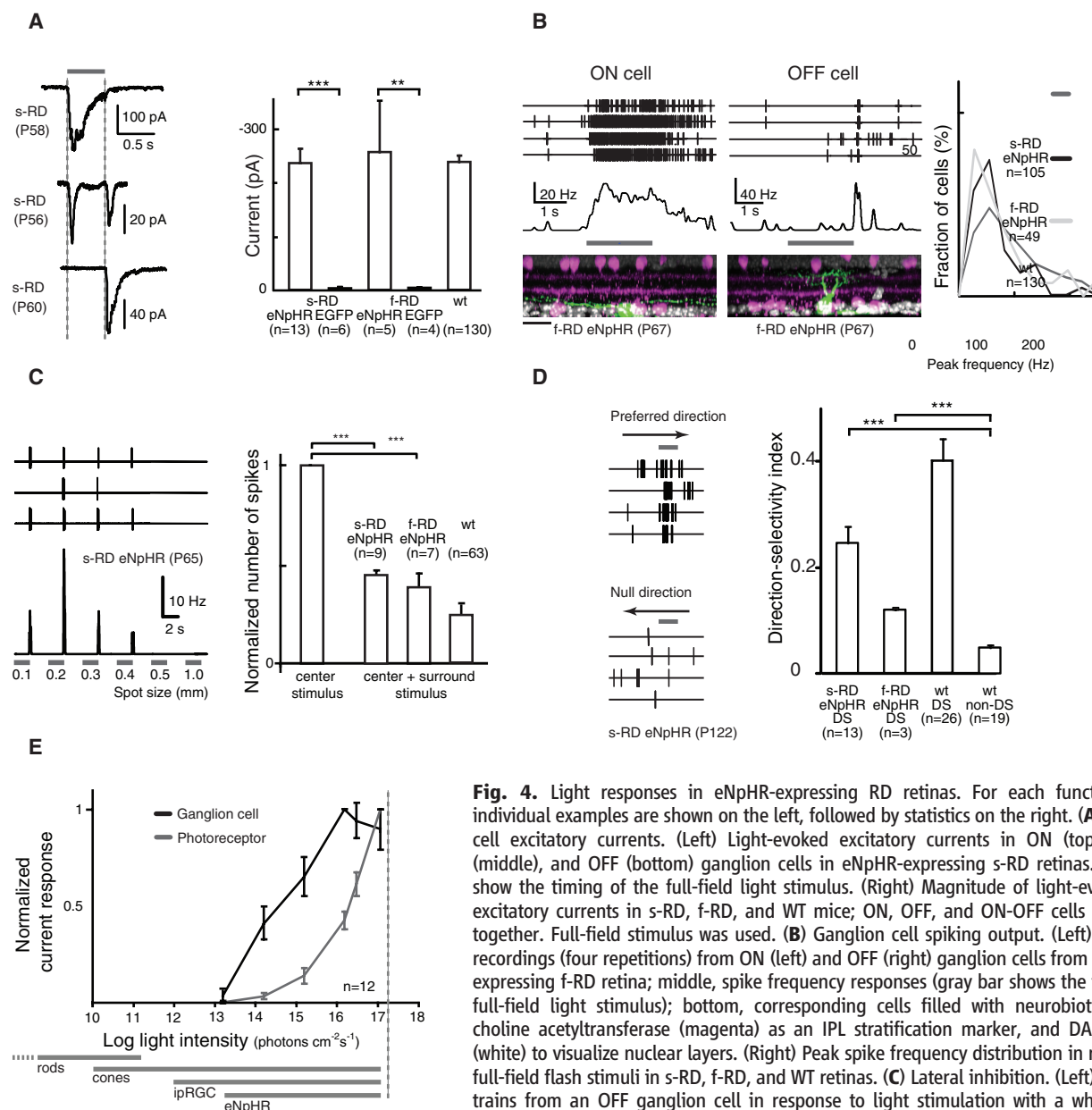


Fig. 4. Light responses in eNpHR-expressing RD retinas. For each functional test, individual examples are shown on the left, followed by statistics on the right. **(A)** Ganglion cell excitatory currents. (Left) Light-evoked excitatory currents in ON (top), ON-OFF (middle), and OFF (bottom) ganglion cells in eNpHR-expressing s-RD retinas. Gray bars show the timing of the full-field light stimulus. (Right) Magnitude of light-evoked peak excitatory currents in s-RD, f-RD, and WT mice; ON, OFF, and ON-OFF cells are pooled together. Full-field stimulus was used. **(B)** Ganglion cell spiking output. (Left) Top, spike recordings (four repetitions) from ON (left) and OFF (right) ganglion cells from an eNpHR-expressing f-RD retina; middle, spike frequency responses (gray bar shows the timing of a full-field light stimulus); bottom, corresponding cells filled with neurobiotin (green), choline acetyltransferase (magenta) as an IPL stratification marker, and DAPI staining (white) to visualize nuclear layers. (Right) Peak spike frequency distribution in response to full-field flash stimuli in s-RD, f-RD, and WT retinas. **(C)** Lateral inhibition. (Left) Top, spike trains from an OFF ganglion cell in response to light stimulation with a white spot of increasing size (three repetitions); bottom, corresponding spike frequency. (Right) Number of spikes evoked by 1-mm-diameter spot stimuli (center + surround stimulus) relative to the number of spikes evoked by a 0.1- to 0.2-mm spot (center stimulus). **(D)** Directional selective responses. (Left) Spike recordings during stimulation with a fast-moving bar (width, 200 μ m; speed, 1.5 mm s⁻¹; four repetitions) in the preferred direction (top) and in the opposite direction ("null" direction, bottom). Stimulus timing is shown by gray bars. (Right) Direction-selectivity index (35, 37). **(E)** Light sensitivity. eNpHR-induced excitatory current responses in ganglion cells and photocurrents in photoreceptors as a function of light intensity. The gray lines at the bottom display the ranges of sensitivities for rods (only partially shown), cones, and intrinsically photosensitive ganglion cells (ipRGCs). The maximum light intensity at 580 nm allowed in the human eye, according to the 2006 European directives on artificial optical radiation (28), is shown by the vertical dashed line. In all panels, n , number of different cells from which we took our measurements; error bars, SEM; stars, statistical significance (35). Light intensity was 10^{16} photons cm⁻² s⁻¹ for each experiment.

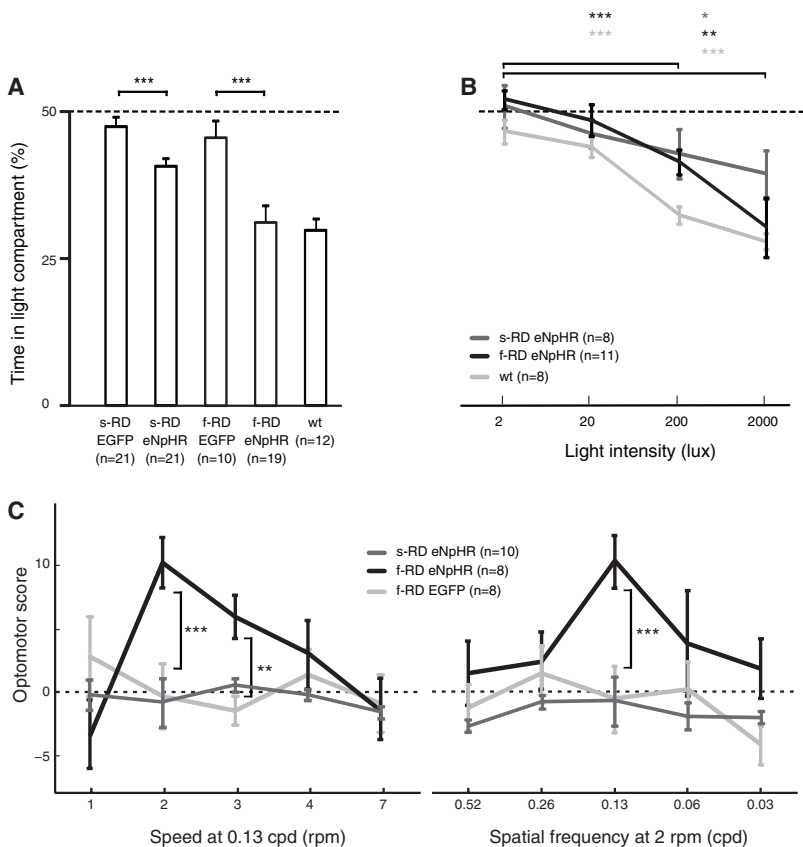


Fig. 5. Visually guided behavior in eNpHR-expressing RD mice. **(A)** Dark-light box experiment. Percentage of time the AAV-injected RD animals and WT mice spent in the light compartment. **(B)** Dark-light box test. Percentage of time mice spent in the light compartment as a function of light intensity. **(C)** Optomotor response score of eNpHR- or EGFP-expressing RD animals at different rotational speeds and spatial frequencies. rpm, revolutions per minute. In all panels, n, number of different animals; error bars, SEM; stars, statistical significance (35).

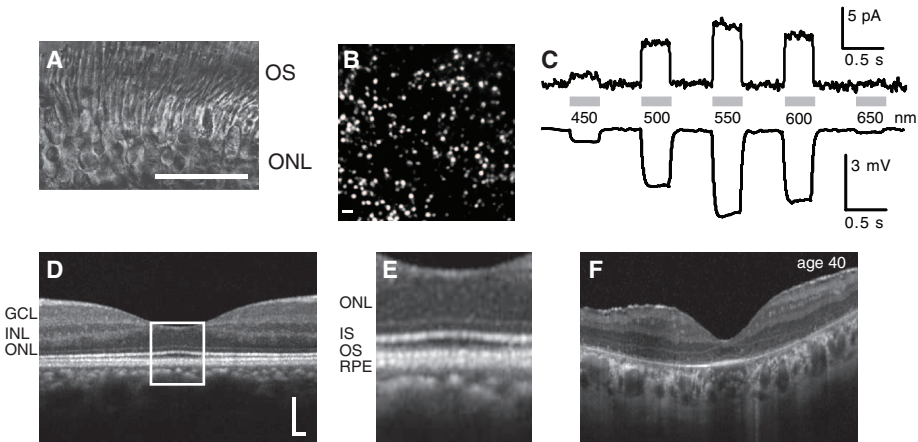


Fig. 6. Translational aspects of eNpHR-EYFP-mediated reactivation of photoreceptors. **(A)** Retinal slice from a human retinal explant (24 hours postmortem). Scale bar, 30 μ m. **(B)** Fluorescent live image of a lentivirus-transfected area from a human retina after 7 days in culture and 2 days after lentiviral administration. Scale bar, 20 μ m. **(C)** Action spectrum of an eNpHR-expressing human photoreceptor stimulated with full-field light flashes ranging from 450 to 650 nm (top, current response; bottom, voltage response). Gray bars indicate the timing of the stimulus. **(D)** Representative OCT scan covering the foveal region of a healthy individual. **(E)** Magnified image. IS, inner segments; RPE, retinal pigment epithelium. Scale bar, 200 μ m. **(F)** OCT from the left eye of a 40-year-old male patient with sporadic retinitis pigmentosa (loss of vision since the age of 15). Outer segments are undetectable.

the retina (22) and the input signal to higher brain centers. Notably, light stimulation evoked excitatory currents and spiking activity in ganglion cells of eNpHR-transduced RD retinas, either at light increments, light decrements, or both (P53 to P264; Fig. 4, A and B). The dendritic processes of morphologically reconstructed ganglion cells were properly aligned in the corresponding ON or OFF strata (23) of the IPL (Fig. 4B). Ganglion cell spike frequencies spanned similar ranges in s-RD, f-RD, and WT retinas (Fig. 4B). Some ganglion cell types respond preferentially to changes in illumination and therefore spike transiently after a light step, whereas others represent the level of illumination and thus spike in a more sustained manner. The dynamics of spiking activity in eNpHR-transduced retinas varied from transient to sustained, as in WT retinas (fig. S7). Light-stimulation of eNpHR-reactivated photoreceptors in RD retinas even evoked ganglion cell spiking activity at later stages of degeneration (s-RD: <P255, f-RD: <P264; fig. S7).

We next asked if basic forms of spatial processing were functional in the eNpHR-transduced RD retinas. Lateral inhibition is a conserved feature of vertebrate retinas that is important for spatial contrast (“edge”) enhancement. When spots of increasing sizes were presented to eNpHR-expressing RD retinas, the response magnitude of ganglion cells reached a maximum and then gradually decreased, a sign of lateral inhibition (Fig. 4C and fig. S7) (21, 24). Lateral inhibition also results in ON-center, OFF-surround responses (21) that we were able to observe in eNpHR-expressing RD retinas (fig. S7). Another example of spatial processing is the directional-selective responses of types of ganglion cells to motion stimuli (25). The activity of directional selective ganglion cells is important for the optokinetic reflex (26). When eNpHR-transduced RD retinas were stimulated with bars moving in different directions, some ganglion cells responded preferentially to motion in a particular direction but produced little activity when the bar moved in the opposite direction (Fig. 4D) (27). This response asymmetry suggests that the retinal circuit for directional selectivity is, at least partially, maintained in RD retinas.

The sensitivity of eNpHR to light is less than that of normal cones (Fig. 4E), and although normal cones can adapt to different light intensities, eNpHR-driven cones have a fixed sensitivity range (see SOM). However, the sensitivity of ganglion cells to light was 1.7 log units higher than that of the eNpHR-expressing photoreceptors (Fig. 4E), and the light levels required for eNpHR stimulation at 580 nm are below the limit allowed for safe radiation of the human eye, according to the 2006 European directives on artificial optical radiation (Fig. 4E) (28).

The slow and fast RD mouse models differ not only in the time course of photoreceptor degeneration, but also in the amount of light-driven activity present during development. s-RD mice

have no light-sensitive rod-cone system during development (15), whereas f-RD mice lose the rod-cone function gradually and are blind by 4 weeks of age (16). In the retina, the responses in eNpHR-activated s-RD and f-RD mice were similar, suggesting that the development of the tested retinal functions, like direction selectivity, may not require light-driven input from rods and cones. Light stimulation of the eyes resulted in visually evoked potentials in eNpHR-expressing f-RD mice but not in EGFP-transduced controls (P42 to P118, fig. S8). In contrast to the retina, we could not measure light-driven cortical activity in eNpHR-transduced s-RD mice.

We next evaluated whether light could induce behavioral changes in eNpHR-transduced mice. In dark-light box tests (29), eNpHR-expressing f-RD and s-RD mice performed significantly better than the corresponding EGFP-expressing control groups (P44 to P143, Fig. 5A), and the increased performance depended on the illumination level (Fig. 5B). In the optomotor reflex test (30), only eNpHR-expressing f-RD mice performed better than EGFP-expressing control mice at a variety of drum speeds (P69 to P153, Fig. 5C). WT (fig. S8) and f-RD responses both peaked at the same speed. The optimum spatial frequency was higher for WT animals [0.26 cycles per degree (cpd)] compared to f-RD (0.13 cpd). These experiments demonstrated that resensitized photoreceptors are able to drive visually guided behavior in f-RD mice and, to some extent, in s-RD mice.

To test for potential toxicity of eNpHR or the unmodified NpHR, we first compared the retinas of eNpHR-transduced WT mice (6 weeks after AAV administration) with those of normal WT mice. The number of photoreceptors was similar in both conditions (fig. S9), and, in addition to the light-induced spiking activity, ganglion cells in eNpHR-transduced WT retinas had a wider action spectrum (a gain of function at longer wavelengths) than ganglion cells in uninjected WT retinas (fig. S9). This suggests that both intrinsic opsins and eNpHR are at work. Next, we compared the retinas of transgenic mice expressing NpHR in photoreceptors under the control of a bovine rhodopsin promoter with WT retinas and found similar numbers of photoreceptors (at P140, fig. S9). These results suggest that, in the studied time window, neither the unmodified NpHR nor eNpHR induced additional photoreceptor degeneration.

The translation of gene therapy achieved in mice to human participants requires the use of promoters and AAV serotypes that drive photoreceptor-specific eNpHR expression in human retinas. Therefore, we tested our AAVs on human ex vivo retinal explants (Fig. 6A), which we could keep in culture for 2 to 3 weeks. Because of this short time window and the relatively long period of time required to efficiently express eNpHR from AAVs, we had to use immunohistochemistry to visualize eNpHR-EYFP protein expression in the cultured human retinas. Of the three promoters, mCAR directed expression of eNpHR specifi-

cally in human photoreceptors (fig. S10). To reduce the time required to obtain robust eNpHR-EYFP expression, which is necessary for two-photon laser-targeted electrophysiology, we inserted the mCAR-eNpHR construct into a lentiviral vector (fig. S10) (31). Using this new vector, we found high levels of eNpHR expression, specifically in human photoreceptors after only 1 to 2 days of incubation (Fig. 6B and fig. S10). Brightly labeled photoreceptors in the parafoveal region displayed photocurrents and photovoltages with spectral tuning reflecting eNpHR activation (Fig. 6C and fig. S10). We could not measure any photocurrents from control human retinas, even at the time when the retina was isolated.

To find potential patients with retinitis pigmentosa eligible for eNpHR-mediated restoration of visual functions, we screened a database (see SOM) that contained records of retinal images acquired by optical coherence tomography (OCT), Goldman visual field tests, multifocal electroretinograms (ERGs), full-field ERGs, and visual-acuity tests. We identified legally blind patients (data from one of whom are shown in Fig. 6 and fig. S11) with no visible outer segments on OCT pictures, but cone cell bodies in the central region. These criteria may be used in the future for selecting patients who could benefit from this therapy.

We have shown that a microbial gene introduced to surviving cone cell bodies reactivated retinal ON and OFF pathways and the retinal circuitry for lateral inhibition and directional selective responses. Moreover, the reactivated cones enabled RD mice to perform visually guided behaviors. The tested time window of intervention was up to ~260 days in f-RD and s-RD mice, suggesting that persisting cone cell bodies (~25%) are enough to induce ganglion cell activity, even during later stages of degeneration. Our finding that AAVs with the mCAR promoter specifically transduced human photoreceptors and the identification of patients with little measurable visual function and no outer segments but surviving cone cell bodies suggest a potential for translating eNpHR-based rescue of visual function to humans (see SOM). In the future, eNpHR-based restoration may be combined with other approaches that increase the survival of altered photoreceptors (20, 32–34).

References and Notes

1. K. Shintani, D. L. Shechtman, A. S. Gurwood, *Optometry* **80**, 384 (2009).
2. T. Cronin, T. Léveillard, J. A. Sahel, *Curr. Gene Ther.* **7**, 121 (2007).
3. R. E. Marc, B. W. Jones, C. B. Watt, E. Strettoi, *Prog. Retin. Eye Res.* **22**, 607 (2003).
4. Z. Y. Li, I. J. Kljavin, A. H. Milam, *J. Neurosci.* **15**, 5429 (1995).
5. B. Lin, R. H. Masland, E. Strettoi, *Exp. Eye Res.* **88**, 589 (2009).
6. A. H. Milam, Z. Y. Li, R. N. Fariss, *Prog. Retin. Eye Res.* **17**, 175 (1998).
7. E. Banin et al., *Neuron* **23**, 549 (1999).
8. E. Bamberg, J. Tittor, D. Oesterhelt, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 639 (1993).
9. B. Schobert, J. K. Lanyi, *J. Biol. Chem.* **257**, 10306 (1982).

10. A. Seki et al., *Biophys. J.* **92**, 2559 (2007).
11. V. Gradinaru, K. R. Thompson, K. Deisseroth, *Brain Cell Biol.* **36**, 129 (2008).
12. F. Zhang et al., *Nature* **446**, 633 (2007).
13. G. P. Gao et al., *Proc. Natl. Acad. Sci. U.S.A.* **99**, 11854 (2002).
14. C. Leberherz, A. Maguire, W. Tang, J. Bennett, J. M. Wilson, *J. Gene Med.* **10**, 375 (2008).
15. E. Claes et al., *Invest. Ophthalmol. Vis. Sci.* **45**, 2039 (2004).
16. D. B. Farber, J. G. Flannery, C. Bowes-Rickman, *Prog. Retin. Eye Res.* **13**, 31 (1994).
17. M. Allocca et al., *J. Virol.* **81**, 11372 (2007).
18. Y. Wang et al., *Neuron* **9**, 429 (1992).
19. X. Zhu et al., *FEBS Lett.* **524**, 116 (2002).
20. C. Punzo, K. Kornacker, C. L. Cepko, *Nat. Neurosci.* **12**, 44 (2009).
21. S. W. Kuffler, *J. Neurophysiol.* **16**, 37 (1953).
22. T. Gollisch, M. Meister, *Neuron* **65**, 150 (2010).
23. E. V. Famiglietti Jr., H. Kolb, *Science* **194**, 193 (1976).
24. H. B. Barlow, *J. Physiol.* **119**, 69 (1953).
25. J. B. Demb, *Neuron* **55**, 179 (2007).
26. K. Yoshida et al., *Neuron* **30**, 771 (2001).
27. H. B. Barlow, R. M. Hill, *Science* **139**, 412 (1963).
28. P. Degenar et al., *J. Neural Eng.* **6**, 035007 (2009).
29. M. Bourin, M. Hascoët, *Eur. J. Pharmacol.* **463**, 55 (2003).
30. J. Abdeljalil et al., *Vision Res.* **45**, 1439 (2005).
31. R. Zufferey, D. Nagy, R. J. Mandel, L. Naldini, D. Trono, *Nat. Biotechnol.* **15**, 871 (1997).
32. B. Chen, C. L. Cepko, *Science* **323**, 256 (2009).
33. T. Léveillard, J. A. Sahel, *Sci. Transl. Med.* **2**, 26ps16 (2010).
34. Y. Yang et al., *Mol. Ther.* **17**, 787 (2009).
35. Materials and methods are available as supporting material on Science Online.
36. S. S. Nikonov, R. Kholodenko, J. Lem, E. N. Pugh Jr., *J. Gen. Physiol.* **127**, 359 (2006).
37. W. R. Taylor, D. I. Vaney, *J. Neurosci.* **22**, 7712 (2002).
38. We thank B. G. Scherf, S. Djaffer, and H. Kohler for technical assistance and P. Lagali, K. Farrow, A. Matus, and S. Oakeley for their comments on the manuscript. pAAV2.1-Rho-EGFP was provided by A. Auricchio, and J. Nathans provided the pRed2.1lacZ. Post-mortem human retinas were provided by T. Wesseling and E. Pels at the Cornea Bank in Amsterdam (Netherlands). This study was supported by Friedrich Miescher Institute funds; a U.S. Office of Naval Research Naval International Cooperative Opportunities in Science and Technology Program grant; a Marie Curie Excellence grant and a European Union (EU) HEALTH-F2-223156 grant to B.R.; a grant from the EU (RETICIRC) to B.R. and S.P.; grants from the Agence nationale de la recherche (MEDINAS, RETINE) to S.P.; a Center Grant from Foundation Fighting Blindness (U.S.) to S.M.-S. and J.A.S.; grants from the Swiss National Science Foundation and the EU to D.T.; a grant from the EU (TREATRUSH) to J.A.S., S.P., and B.R.; a Marie Curie Postdoctoral Fellowship to D.B.; and a National Centers of Competence in Research Frontiers in Genetics fellowship to V.B. and A.C.G. The Ocular Genetics Unit at Trinity College Dublin is supported by Science Foundation Ireland. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession no. GSE22338 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22338).

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1190897/DC1
Materials and Methods
SOM Text
Figs. S1 to S11
Tables S1 to S4
References and Notes

14 April 2010; accepted 11 June 2010
Published online 24 June 2010;
10.1126/science.1190897
Include this information when citing this paper.

Ruling Out Multi-Order Interference in Quantum Mechanics

Urbasi Sinha,^{1*} Christophe Couteau,^{1,2} Thomas Jennewein,¹
Raymond Laflamme,^{1,3} Gregor Weihs^{1,4*}

Quantum mechanics and gravitation are two pillars of modern physics. Despite their success in describing the physical world around us, they seem to be incompatible theories. There are suggestions that one of these theories must be generalized to achieve unification. For example, Born's rule—one of the axioms of quantum mechanics—could be violated. Born's rule predicts that quantum interference, as shown by a double-slit diffraction experiment, occurs from pairs of paths. A generalized version of quantum mechanics might allow multipath (i.e., higher-order) interference, thus leading to a deviation from the theory. We performed a three-slit experiment with photons and bounded the magnitude of three-path interference to less than 10^{-2} of the expected two-path interference, thus ruling out third- and higher-order interference and providing a bound on the accuracy of Born's rule. Our experiment is consistent with the postulate both in semiclassical and quantum regimes.

Born's interpretation (1) of the wave function $\psi(\mathbf{r}, t)$ for a quantum mechanical state stipulates that the probability density to find a particle at position $\mathbf{r}$ and at time t is given by

$$P(\mathbf{r}, t) = \psi^*(\mathbf{r}, t)\psi(\mathbf{r}, t) = |\psi(\mathbf{r}, t)|^2 \quad (1)$$

A double-slit diffraction experiment is a direct consequence of this rule; the probability to detect a particle at $\mathbf{r}$ after passing through an aperture with two slits, A and B , is given by

$$\begin{aligned} P_{AB}(\mathbf{r}) &= |\psi_A(\mathbf{r}) + \psi_B(\mathbf{r})|^2 \\ &= |\psi_A|^2 + |\psi_B|^2 + \psi_A^* \psi_B + \psi_B^* \psi_A \\ &= P_A + P_B + I_{AB} \end{aligned} \quad (2)$$

where we have omitted the position argument for brevity and defined P_i to be the probability with only slit i ($i = A, B$) open. The corresponding (second-order) interference term can be defined as

$$I_{AB} := P_{AB} - (P_A + P_B) = P_{AB} - P_A - P_B \quad (3)$$

Within quantum mechanics, adding more paths (i.e., slits) does not add higher complexity. For three slits A , B , and C (Fig. 1), we find

$$P_{ABC} = P_A + P_B + P_C + I_{AB} + I_{AC} + I_{BC} \quad (4)$$

Therefore, by Born's rule and its square exponent (Eq. 1), interference always occurs in pairs of

possibilities and is defined as the deviation from the classical additivity of the probabilities of mutually exclusive events (2). These possibilities can be associated with any degree of freedom, such as spatial paths, energetic states, angular momentum states, etc. Even if multiple particles are involved, interference occurs in pairs of possibilities. Consequently, we define the third-order interference term I_{ABC} for a three-path configuration (mutually exclusive) as the deviation of P_{ABC} from the sum of the individual probabilities and the second-order interference terms:

$$\begin{aligned} I_{ABC} &:= P_{ABC} - (P_A + P_B + P_C + I_{AB} + I_{BC} + I_{AC}) \\ &= P_{ABC} - P_{AB} - P_{BC} - P_{AC} + P_A + P_B + P_C \end{aligned} \quad (5)$$

A physical system with such probability terms is three-path interference of a photon sent through a mask with three slits (Fig. 1). Note that the definitions in Eq. 3 and Eq. 5 are the first terms in an infinite hierarchy of interference terms (2).

The nonzero interference term I_{AB} is expected in all wave theories, including quantum mechanics (3, 4). The next higher-order (i.e., three-path) interference term I_{ABC} will be zero in all wave theories, with a square-law relation between the field energy (or probability density) and field amplitude, which is the case in quantum mechanics with Born's rule. Moreover, if there is no interference at a certain level in the hierarchy, the higher-order terms must vanish as well (2).

Our aim is to establish experimentally whether the value of I_{ABC} is different from zero. We measure all seven probability terms of Eq. 5 plus the probability P_0 of detecting particles when all slits are closed. P_0 represents the probability of the empty set in an abstract definition, or a background signal in the experiment. The eight terms

are obtained by sending optical photons through three slits, which can be opened or closed individually (see Fig. 1 for the slit details and Fig. 2 for the setup). A double-slit experiment could be used to test Born's rule, but then one would have to measure the nonzero double-slit interference term and compare it with the theoretical prediction. This would be sensitive to experimental parameters such as slit dimensions, wavelength of incident photons, and distance between detector and slits, each with its attendant error. In contrast, we expect the three-path interference term I_{ABC} to be zero, with the advantage of being independent of many experimental parameters, thus enabling a more precise null test for Born's rule.

We measure the terms in Eq. 5 as well as P_0 , which accounts for the inevitable detector noise and background signal. The measured quantity ϵ based on Eq. 5 is given by

$$\epsilon = P_{ABC} - P_{AB} - P_{AC} - P_{BC} + P_A + P_B + P_C - P_0 \quad (6)$$

Here, $p \propto P$ of Eq. 5 and refers to the measured number of photons (or optical intensity, proportional to the photon number) in the various slit combinations. To give a scale to the size of a potential deviation from Born's rule, we define a normalized variant of ϵ called κ (Fig. 3),

$$\kappa \equiv \frac{\epsilon}{\delta} \quad (7)$$

where

$$\begin{aligned} \delta &= |I_{AB}| + |I_{BC}| + |I_{AC}| \\ &= |P_{AB} - P_A - P_B + P_0| + |P_{BC} - P_B - P_C + P_0| + |P_{AC} - P_A - P_C + P_0| \end{aligned} \quad (8)$$

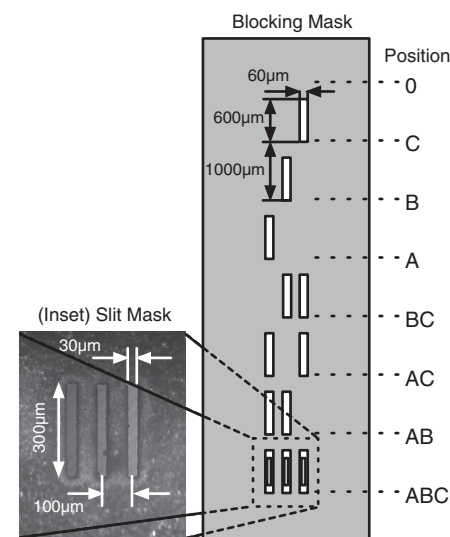


Fig. 1. Arrangement and dimensions of the slits used in the experiment. The blocking mask has open apertures depending on the measured slit combination according to Eq. 6. Inset is an image of the triple-slit mask.

¹Institute for Quantum Computing and Department of Physics and Astronomy, University of Waterloo, 200 University Avenue West, Waterloo, Ontario N2L 3G1, Canada. ²Laboratoire de Nanotechnologie et d'Instrumentation Optique, Université de Technologie de Troyes, 12 rue Marie Curie, 10 000 Troyes, France. ³Perimeter Institute for Theoretical Physics, 31 Caroline Street North, Waterloo, Ontario N2L 2Y5, Canada. ⁴Institut für Experimentalphysik, Universität Innsbruck, Technikerstraße 25, 6020 Innsbruck, Austria.

*To whom correspondence should be addressed. E-mail: usinha@iqc.ca, gregor.weihs@uibk.ac.at

Here, δ is the sum of the absolute values of the double-slit interference terms, and κ can be seen as the ratio of an unexpected three-path interference term to the expected two-path interference term. If $\delta = 0$, then $\varepsilon = 0$ trivially, and one deals with classical probabilities instead of quantum behavior. Thus, a nonzero δ ensures that we are in a quantum mechanical regime. In an experiment, we never measure probabilities directly, but only absolute frequencies of photon occurrences. The quantity κ is independent of the total particle flux onto the slits as long as it is constant in time.

To measure κ in various optical power regimes, we used different types of photon sources. Figure 2 shows details of the experimental setup. We used a laser attenuated to a power level of a few microwatts down to ~ 200 fW (single-photon level) as well as heralded single photons ($\sim 40,000$ photons/s) created by spontaneous parametric downconversion (5).

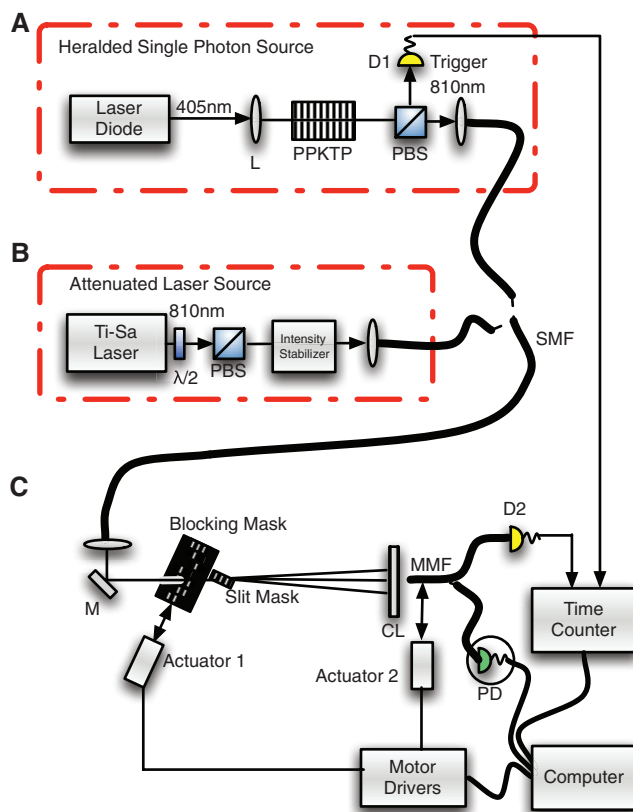
At the photon-counting level, the detection mechanism is based on a silicon avalanche photodiode (APD), and thus the particle-like nature of light is incorporated in the experiments. At the

microwatt level, a series of measurements was performed with a standard optical power meter, using a silicon photodiode. The power meter measurements investigated the optical regime in which particle character is not of concern. In all cases we performed a large number of measurements at fixed points in the diffraction pattern [fig. S1 in (5)]. In addition, we have also performed measurements to check the variation of κ as a function of detector position. Born's rule would predict that κ should be independent of detector position. However, systematic errors may vary with the position and therefore are seen to bring a variation in the measured value of κ at different detector positions even in our experiment. Nonetheless, the mean κ is within the bounds set by the attendant errors at each such detector position.

The typical distributions of measured values of κ are shown in Fig. 3, with photon streams from a laser attenuated to different levels (Fig. 3, A and B) and from a heralded single-photon source (Fig. 3C). κ is calculated from the measured interference intensities for the eight independent slit combinations at a fixed position.

Fig. 2. Experimental set-up used for the measurement of κ .

(A) Creation of heralded single photons from a periodically poled potassium titanyl phosphate (PPKTP) nonlinear crystal pumped by a 405-nm laser diode. Parametric downconverted photons are emitted as pairs at 810 nm and are coupled into a single-mode fiber (SMF). Photon detection (D1) in the trigger output heralds a single photon, which is then sent through the diffraction slits. (B) A pulsed titanium-sapphire (Ti-Sa) laser is attenuated and coupled into a SMF. The attenuation is realized by the combination of a half-wave plate ($\lambda/2$) and a polarizing beamsplitter (PBS), combined with neutral filters and an intensity stabilizer. (C) Schematic of the three-slit experiment where the photons from the source go through the movable blocking mask with the eight combinations and then through



the slit mask, which has the three slits cut into it. We keep the slit mask stationary, whereas the blocking mask consists of bigger and wider slits that open up the various slit combinations as it moves up and down. In this way, we ensure that the same set of slits is used for measuring the different combinations, thus eliminating any dependence on the slit properties. The diffracted light is condensed vertically with a cylindrical lens (CL) onto a multimode fiber (MMF, core size $62.5 \mu\text{m}$), ~ 180 mm from the slits. This fiber (movable along the diffraction pattern) acts as an aperture to probe the interferences. The collected photons are detected either with an avalanche photodiode (D2) whose signals are recorded with a time counter, or with an optical power meter (PD), both connected to a computer. For heralded single photons, detections are conditioned on the detection of a trigger photon.

The order of the eight slit combinations was chosen randomly for reducing systematic influences on κ caused by slow variations of the photon flux. Each combination in a run was measured for a certain photon-count integration time, and up to 100 runs were cycled to obtain a statistically significant sample of κ values. Among the many positions in the diffraction pattern, we chose the central maximum of the triple-slit combination (yielding the maximum number of coincidence photon counts) to obtain our data (5). For the single-photon source, we measured at each slit combination until the trigger count reached 30 million, which was a good compromise between accumulating a statistically significant number of coincidences for the different slit combinations and ensuring a low drift of the photon source between measurements.

With a null experiment, a very careful analysis of random and systematic errors must be undertaken, as our bound on the amount of three-path interference will be directly related to the level of experimental uncertainty. Among the random errors in our setup, thermal and acoustic fluctuations cause the source fluxes to vary in time. In addition, detection efficiency and optical alignment can change. In particular, there will be some mechanical vibration of the thin ($25 \mu\text{m}$) slotted steel membrane apertures, causing a variable slit transmission due to near-field diffraction. In addition, for power meter measurements, the instrumental error is added to the above error sources, whereas for photon counting, the Poissonian distributed counting error is the dominating fluctuation. Because of the random nature of the individual errors, we used Gaussian error propagation to estimate the error of κ , where we used the standard variances of the individual measurement values calculated from a large number of repetitions of the experiments. In some cases where we observed a drift in the rates, we found the Allen variance of the values to be a better estimator for error propagation. This is justifiable because κ is calculated from eight measurements taken in direct succession, and the variance between subsequent samples of each quantity p_A , p_B , etc., is therefore the most suitable error estimator.

Once we understand the random errors, we can characterize the systematic errors. Our experiment and the measurement of κ are convenient, as they neither require the slits to be identical nor require the transmission values to be perfectly 1 and 0. On the other hand, what matters is the absence of correlation or systematic variation in how the slits behave while switching between slit combinations. Note that the size of the slits and the wavelength make independent shutters difficult to insert, and we used a static opening mask plate in front of the actual slits for blocking and unblocking the individual slits.

Our approach can potentially introduce unwanted correlations between the switching of different combinations. This occurred in our case; a fault in the blocking mask in the BC combination caused opening B to be shifted off its nominal

position by 8 μm . At zero distance between opening and slit masks, this shift would not affect the transmittance of the diffracting slit. However, with the finite separation of 50 μm between the two masks, it does matter. Using a two-dimensional finite-difference time domain simulation of the light field between the wider opening and narrower slit, we found that this lateral misalignment could change the effective slit transmission by $\pm 3\%$, depending on the relative position. Using this to adjust the slit transmittance value for slit *B* in the combination *BC* leads to a value of $|\kappa| = 0.01$. Bringing the masks closer would reduce this error, but the thin membranes tend to stick together when they come closer than 50 μm .

Another major systematic error is detector nonlinearity. There is no perfect detector, and efficiency and nonlinearity will always be finite. For our commercial power meter, the nonlinearity is 0.5% for all ranges. For the worst case, at the central maximum of the diffraction pattern, this

results in a systematic error of $|\kappa| = 0.003$. For photon-counting detectors, there is always a finite dead-time during which they are blind to photons. Depending on the flux, this results in a saturation effect; that is, the detector response does not follow the square law, but has a deviation potentially indistinguishable from a violation of Born's rule. Therefore, to keep the nonlinearity error negligible, we used count rates below 100,000/s.

Combining the various error sources, our particular setup enables us to bound the measurements of κ to better than 0.01, thus providing a bound on the accuracy of Born's rule. This is in good agreement with the values measured for single photons, $\kappa = 0.0064 \pm 0.0120$, and for the attenuated laser beams, $\kappa = 0.0073 \pm 0.0018$ (power meter measurement) and $\kappa = 0.0034 \pm 0.0038$ (APD measurement) from Fig. 3.

Note that any significant nonzero observation of I_{ABC} would imply that Born's rule does not strictly hold. The consequences of detecting even

a small amount of three-way interference (by deviating from the quantum mechanical null prediction) would be tremendous. A modification to Born's rule that leads to multi-order interference would have repercussions on the allowable dynamics. In particular, if probability must be conserved, then Schrödinger's equation would likely have to be modified as well. Nonlinear extensions to quantum mechanics are one way to generalize it (6–8), and there have been efforts to test these nonlinearities (9–12). However, in such experiments, a model was assumed for the nonlinear variant of the Schrödinger equation, and efforts were concentrated on estimating the coefficient of the nonlinearity. In contrast, we present a dedicated test for Born's rule and are able to confirm it within our experimental limitations without depending on specific nonlinear extensions of quantum mechanics.

We are able to bound the magnitude of the third-order interference term to less than 10^{-2} of the regular expected second-order interference, at several detector positions. Thus, our experiment is able to rule out the existence of third-order interference terms (and, in effect, any higher-order interference terms) up to this bound. This bound on the accuracy of Born's rule is relevant for theoretical attempts to derive it (13) as well as for the generalization of quantum mechanics. Among the consequences of such a generalized theory, it would require more detailed specifications of the quantum system (14) and could modify computational complexity by allowing one to distinguish between orthogonal states, thus breaking quantum cryptography and making quantum computing more powerful [i.e., super-quantum computing (15)]. The triple-slit experiment is a simple and very natural system to investigate three-path interference, but it is not the only possible implementation; any configuration of three mutually exclusive quantum paths can be used in such a test. Although our implementation did not lead to the observation of any deviations, such future tests with other systems might indeed lead to improved accuracies on the bound of κ . It would be interesting to perform tests with other types of particles such as neutrons (16, 17), tests of C_{60} molecule interference (18) or electron interference with slits or potential wells (19, 20), or tests of a system with a global wave function, such as a Bose-Einstein condensate (21, 22).

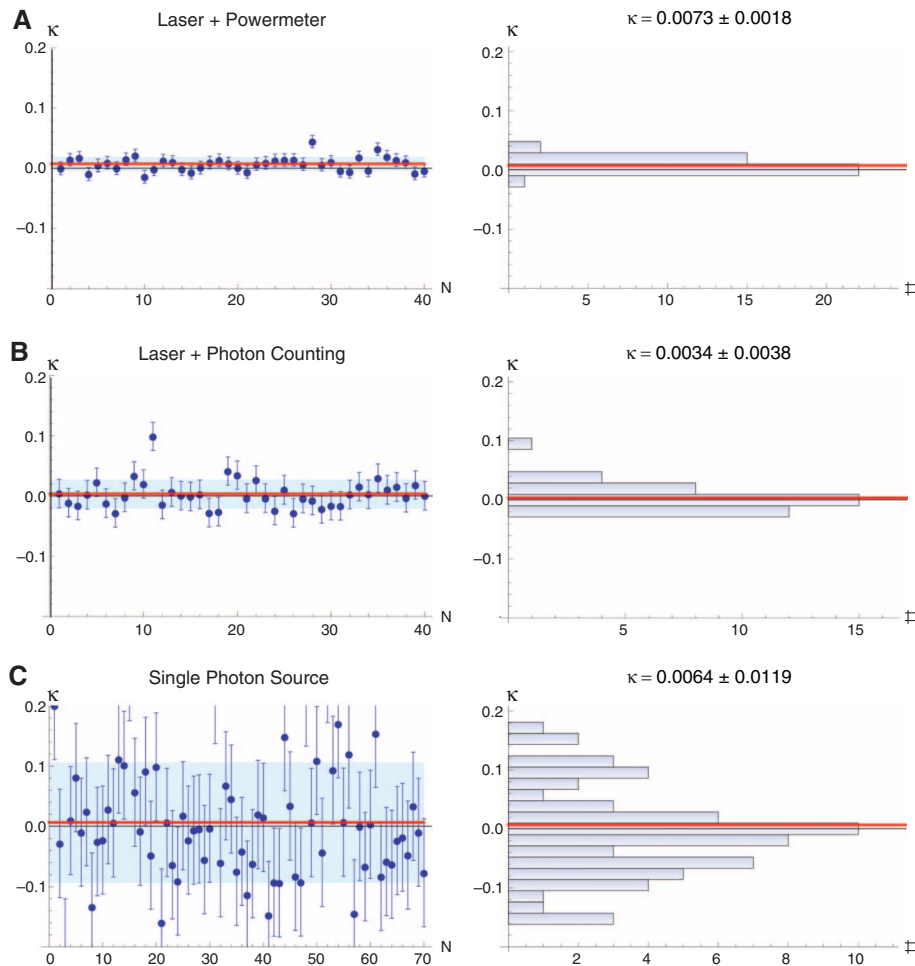


Fig. 3. Typical distributions of κ measured at the central maximum of the triple-slit interference pattern. (A) Data using a laser source and a power meter for detection, resulting in $\kappa = 0.0073 \pm 0.0018$. (B) Data with the laser but attenuated to single-photon level and an APD for detection, resulting in $\kappa = 0.0034 \pm 0.0038$. (C) Data taken using the heralded single-photon source and an APD for detection, resulting in $\kappa = 0.0064 \pm 0.0119$. The Allen variance of κ was used to plot the error bars. The horizontal red lines represent the mean κ values; the blue shaded regions represent a band of one standard deviation of the distribution of κ values around the mean.

References and Notes

1. M. Born, *Z. Phys.* **37**, 863 (1926).
2. R. D. Sorkin, *Mod. Phys. Lett. A* **9**, 3119 (1994).
3. P. Grangier, G. Roger, A. Aspect, *Europhys. Lett.* **1**, 173 (1986).
4. V. Jacques *et al.*, *Eur. Phys. J. D* **35**, 561 (2005).
5. See supporting material on Science Online.
6. S. Weinberg, *Phys. Rev. Lett.* **62**, 485 (1989).
7. I. Bialynicki-Birula, J. Mycielski, *Ann. Phys.* **100**, 62 (1976).
8. C. Simon, V. Buzek, N. Gisin, *Phys. Rev. Lett.* **87**, 170405 (2001).
9. R. Gähler, A. G. Klein, A. Zeilinger, *Phys. Rev. A* **23**, 1611 (1981).
10. J. J. Bollinger, J. D. Prestage, W. M. Itano, D. J. Wineland, *Phys. Rev. Lett.* **54**, 1000 (1985).

11. R. L. Walsworth, I. F. Silvera, E. M. Mattison, R. F. Vessot, *Phys. Rev. Lett.* **64**, 2599 (1990).
12. P. K. Majumder, B. J. Venema, S. K. Lamoreaux, B. R. Heckel, E. N. Fortson, *Phys. Rev. Lett.* **65**, 2931 (1990).
13. W. H. Zurek, *Phys. Rev. Lett.* **90**, 120404 (2003).
14. C. Ududec, H. Barnum, J. Emerson, *Found. Phys.* (2010).
15. S. Aaronson, *Proc. R. Soc. Lond. A* **461**, 3473 (2005).
16. J. Summhammer, H. Rauch, D. Tuppinger, *Phys. Rev. A* **36**, 4447 (1987).
17. A. Zeilinger, R. Gähler, C. Shull, W. Treimer, W. Mampe, *Rev. Mod. Phys.* **60**, 1067 (1988).
18. M. Arndt et al., *Nature* **401**, 680 (1999).
19. A. Ohshita, N. Takayama, H. Tomita, *J. Electron. Microsc.* **34**, 357 (1985).
20. L. Gaudreau et al., *Phys. Rev. Lett.* **97**, 036807 (2006).
21. M. R. Andrews et al., *Science* **275**, 637 (1997).
22. M. Greiner, O. Mandel, T. W. Hänsch, I. Bloch, *Nature* **419**, 51 (2002).
23. U.S., C.C., and T.J. performed the experiments; C.C., R.L., and G.W. conceived of and supervised the experiment and developed the theoretical aspects; G.W. and U.S. performed the modeling and the data and error analysis; and all authors contributed to the writing of the paper. We thank H. Hübel, R. Kaltenböck, A. J. Leggett, A. Sinha, R. D. Sorkin, and A. Zeilinger for valuable

discussions, and Z. Medendorp and I. Söllner for technical assistance earlier in the experiments. Supported by The Natural Science and Engineering Research Council, Canada Foundation for Innovation, European Research Area, Quantum Works, Ontario Centres of Excellence, and Canadian Institute for Advanced Research.

Supporting Online Material

www.sciencemag.org/cgi/content/full/329/5990/418/DC1
Materials and Methods

Fig. S1

References

6 April 2010; accepted 9 June 2010

10.1126/science.1190545

Identification of Carbonate-Rich Outcrops on Mars by the Spirit Rover

Richard V. Morris,^{1*} Steven W. Ruff,² Ralf Gellert,³ Douglas W. Ming,¹ Raymond E. Arvidson,⁴ Benton C. Clark,⁵ D. C. Golden,⁶ Kirsten Siebach,⁴ Göstar Klingelhöfer,⁷ Christian Schröder,⁸ Iris Fleischer,⁷ Albert S. Yen,⁹ Steven W. Squyres¹⁰

Decades of speculation about a warmer, wetter Mars climate in the planet's first billion years postulate a denser CO₂-rich atmosphere than at present. Such an atmosphere should have led to the formation of outcrops rich in carbonate minerals, for which evidence has been sparse. Using the Mars Exploration Rover Spirit, we have now identified outcrops rich in magnesium-iron carbonate (16 to 34 weight percent) in the Columbia Hills of Gusev crater. Its composition approximates the average composition of the carbonate globules in martian meteorite ALH 84001. The Gusev carbonate probably precipitated from carbonate-bearing solutions under hydrothermal conditions at near-neutral pH in association with volcanic activity during the Noachian era.

The existence of carbonate minerals on Mars has long been postulated, based on evidence of past and present water along with a CO₂-rich atmosphere that may have been denser during the Noachian era (1–5). Carbonate minerals have been identified in martian meteorites as minor phases (~1 volume % or less) by petrographic and microbeam methods (5) and detected by orbital observations in regional-scale rock units in Nili Fossae at unknown abundances (6) and, equivocally, in martian dust and in soil as a minor component (<5 volume %) (7–10). Here, we describe the detection of carbonate as a major component in outcrops in the Columbia Hills of Gusev crater.

The Columbia Hills are an “island” of older rocks surrounded by Hesperian-aged olivine-bearing volcanic rocks that dominate the plains within Gusev crater (11). The Hills form a crudely antiformal structure composed of massive-to-layered rocks with varying compositions and extents of aqueous alteration (12–16). The Inner Basin within the

Hills is dominated by volcanoclastic rocks, including Home Plate, and alteration phases (sulfates and opaline silica deposits) of likely hydrothermal origin (17–21). The Mars Exploration Rover Spirit descended from Haskin Ridge on the eastern side of the summit of Husband Hill into the Inner Basin of the Columbia Hills to the eastern edge of the El Dorado ripple field during its second summer at Gusev crater (16). A series of benches (Fig. 1) with olivine-rich outcrops were encountered and analyzed by Spirit's instruments (19, 21). The Comanche outcrops, with their granular surface textures (grain sizes ~0.5 to 1.0 mm), are erosional remnants that are draped over the older and more massive Algonquin and other olivine- and pyroxene-rich outcrops (Figs. 1 and 2). They have layering whose bedding is roughly conformal with local topography, consistent with a volcanoclastic origin, and have fracture-filling deposits that have withstood aeolian erosion better than the surrounding materials, forming raised fins (Figs. 1 and 2).

Spirit's Mössbauer (MB) spectrometer provides information about Fe mineralogy and the distribution of Fe among Fe-bearing phases and oxidation states (22). The MB spectrum of Comanche Spur (Fig. 3A) is characterized by two Fe²⁺ doublets, which were initially assigned to Fe²⁺ in olivine and to either Fe²⁺ in pyroxene atypical of other Gusev pyroxenes or to Fe²⁺ in a phase other than pyroxene (19). The doublet identification diagram (Fig. 3B), which now includes data for Fe²⁺-bearing carbonates (23), shows that

Comanche Spur is an olivine-carbonate assemblage. The MB data indicate the presence of Mg-Fe carbonate in the Comanche outcrops [Fig. 3B and (23)].

In addition to major element chemistry, the Spirit's Alpha Particle X-Ray Spectrometer (APXS) instrument can determine an aggregate concentration for excess light elements through analysis of photon scattering peaks (24, 25). The energy of the APXS scattering photons (14.3 keV) is virtually identical to that for the MB gamma ray (14.4 keV), so that the depth of rock analyzed by the two instruments is equivalent. The average excess light-element concentration of Comanche Spur, measured on surfaces brushed by the Rock Abrasion Tool (RAT), is 12 ± 5 weight percent (wt %) equivalent CO₂ (Table 1). The low bulk CaO concentration of Comanche Spur (1.69 wt %) requires that the carbonate have a low Ca content, consistent with MB data (Fig. 3B). For comparison, the excess light-element concentration of Algonquin is 0 ± 5 wt % equivalent CO₂. The carbonate content of Comanche is equivalent to ~3% C in the sample. Because of CO₂ in the martian atmosphere, this value is close to the expected APXS alpha channel detection limit for C, so we have not been able to use alpha data to detect C specifically.

We calculated the chemical composition of Comanche carbonate and olivine using previously reported APXS data (21), recalculated to include CO₂, and the percentages of Fe associated with those phases from MB data (Fig. 3A). The calculations yielded (Table 1) a Mg-Fe carbonate (Mc_{0.62}Sd_{0.25}Cc_{0.11}Rh_{0.02}, where Mc = magnesite, Sd = siderite, Cc = calcite, and Rh = rhodochrosite) and a forsteritic olivine (Fo_{0.72}Fa_{0.28}, where Fo = forsterite and Fa = fayalite). All Ca and Mn was assigned to the carbonate to give an upper-limit concentration for those elements, but we have no information as to their actual phase association. The calculated carbonate and olivine compositions are Mc_{0.75}Sd_{0.25} and Fo_{0.70}Fa_{0.30} when Ca and Mn are not associated with the carbonate. In either case, the concentration of carbonate in Comanche Spur is ~26 wt % (Table 1). Using the 7.0 to 17.0 wt % range of the CO₂ concentration (Table 1), the range in the carbonate concentration is ~16 to 34 wt %.

Spectra obtained by Spirit's Miniature Thermal Emission Spectrometer (Mini-TES) provide

¹NASA Johnson Space Center, Houston, TX 77058, USA. ²Arizona State University, Tempe, AZ 85287, USA. ³University of Guelph, Guelph, Ontario, Canada. ⁴Washington University in Saint Louis, St. Louis, MO 63130, USA. ⁵Space Sciences Institute, Boulder, CO 80301, USA. ⁶Engineering and Science Contract Group—Hamilton Sundstrand, Houston, TX 77058, USA. ⁷Johannes Gutenberg-Universität, Mainz, Germany. ⁸University of Bayreuth and Eberhard Karls University of Tübingen, Tübingen, Germany. ⁹Jet Propulsion Laboratory, Pasadena, CA 91109, USA. ¹⁰Cornell University, Ithaca, NY 14853, USA.

*To whom correspondence should be addressed. E-mail: richard.v.morris@nasa.gov

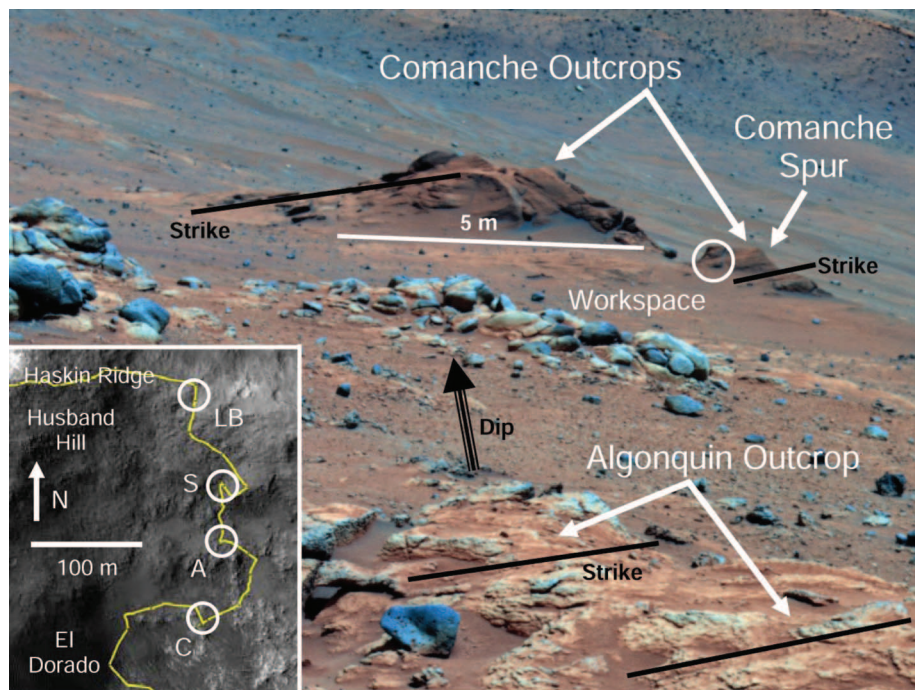


Fig. 1. False-color Pancam image (Sol689_P2571) looking downslope over Algonquin and Comanche outcrops. Strike and dip are indicated by black lines and black arrow. The location of the MB and APXS workspace is indicated by the white circle. The inset locates from high to low elevation the olivine-rich outcrops Larry's Bench (LB), Seminole (S), Algonquin (A), and Comanche (C) on Spirit's traverse across Haskin Ridge down the southeast slope of Husband Hill. The distance between Algonquin and Comanche outcrops is ~85 m. [Credits: NASA/JPL/Pancam and NASA/UA/HiRISE]

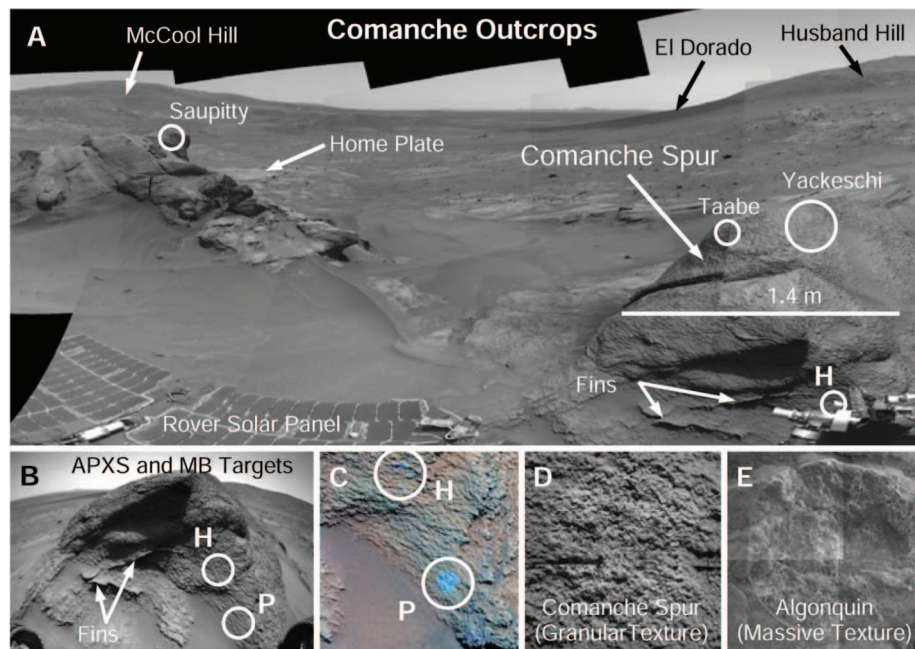


Fig. 2. (A) Navigation Camera (Navcam) mosaic image of the Comanche outcrop. Taabe, Saupitty, and Yackeschi are Mini-TES and Pancam target locations. (B) Hazards Camera (Hazcam) image that locates APXS and MB targets Horseback (H) and Palomino (P) on Comanche Spur. The objects in the lower right and left corners are rover wheels. (C) Pancam false-color image of the ~4.5-cm-diameter RAT-brushed spots (light blue color within white circles) that shows more complete removal of surface dust for the Palomino target. (D) and (E) Microscopic Imager (MI) mosaic of post-brush surfaces (field of view ~4 cm) of Comanche Spur Palomino (D) with a granular surface texture (grain sizes ~0.5 to 1.0 mm) and Algonquin Iroquet (E) with a massive surface texture. [Credit: NASA/JPL/Navcam/Hazcam/Pancam/MI]

information on bulk mineralogy and modal abundance as a function of area in measured targets (26). Mini-TES spectra taken at eight locations on the Comanche outcrops are comparable in spectral shape (Fig. 3C), implying relatively uniform mineralogy across the outcrops. The target Taabe (Fig. 2) is on the same outcrop (Comanche Spur) as the MB and APXS targets, but the Taabe spectrum is compromised by a calibration artifact that affects the high-wave number region ($>1300\text{ cm}^{-1}$; excluded in Fig. 3C) and by its cooler temperature and greater dust contribution as compared to the superior spectrum obtained from target Saupitty on a different Comanche outcrop (Fig. 2).

Three absorption features attributable to fundamental infrared vibrational modes of carbonate are apparent in the Comanche Saupitty spectrum (Fig. 3D). Linear least-squares unmixing calculations (27) of this spectrum require carbonate to fit these features. If carbonates are excluded, the model fit is visually poorer, and the root mean square error doubles (0.15% versus 0.30%). Mg-Fe carbonate and Ca-Mg carbonate (dolomite) are spectrally indistinguishable over the wave-number range used for the calculations. We excluded Ca-Mg carbonate from the final modeling, because Ca-rich carbonates are precluded on the basis of APXS data and are inconsistent with MB results as discussed above. Only three mineralogic components in approximately equal abundance are necessary to provide a good fit: Mg-rich olivine (Fo_{60-80}), an amorphous silicate of basaltic composition, and Mg-Fe carbonate [Fig. 3D (23)].

Multispectral data obtained from the Panoramic Camera (Pancam) for the target Yackeschi (23) show evidence for olivine and nanophase ferric oxide, but Pancam does not cover the spectral region where the Fe^{2+} absorptions of carbonates occur (23).

Comanche carbonate has a well-constrained composition ($\text{Mc}_{0.62}\text{Sd}_{0.25}\text{Cc}_{0.11}\text{Rh}_{0.02}$) and is a major component, based on measurements made on exterior outcrop surfaces at multiple locations by both APXS and MB (~26 wt % with a range of 16 to 34 wt %) and Mini-TES (~34 area %). The carbonate is considered a major volumetric component rather than a surface coating, because the outcrop is an erosional remnant. Martian meteorites have at most ~1 volume % of any carbonate phase, which is heterogeneously distributed and occurs as veins and fracture fills, primarily in the nakhlites (clinopyroxenites) and ALH 84001 (orthopyroxenite) (5, 28, 29). ALH 84001 has the most carbonate, with an average composition [$\text{Mc}_{0.58}\text{Sd}_{0.29}\text{Cc}_{0.12}\text{Rh}_{0.01}$] (28, 30) that is similar to the Comanche carbonate composition. Synthetic analog samples for ALH 84001 carbonate globules, with comparable average compositions [$\text{Mc}_{(0.11-0.72)}\text{Sd}_{(0.16-0.83)}\text{Cc}_{(0.06-0.17)}\text{Rh}_{(0.00-0.02)}$], have the same MB parameters as Comanche carbonate [Fig. 3B and (23)].

Like Comanche and ALH 84001 carbonates, orbital data for martian dust and regional-scale

surfaces in Nili Fossae point to Mg-rich carbonates, although the dust detection (<5 volume % magnesite) has alternatively been interpreted as

sulfate instead of carbonate (7–9). Visible, near-infrared (VNIR) spectra for Nili Fossae are interpreted as magnesite on the basis of two bands

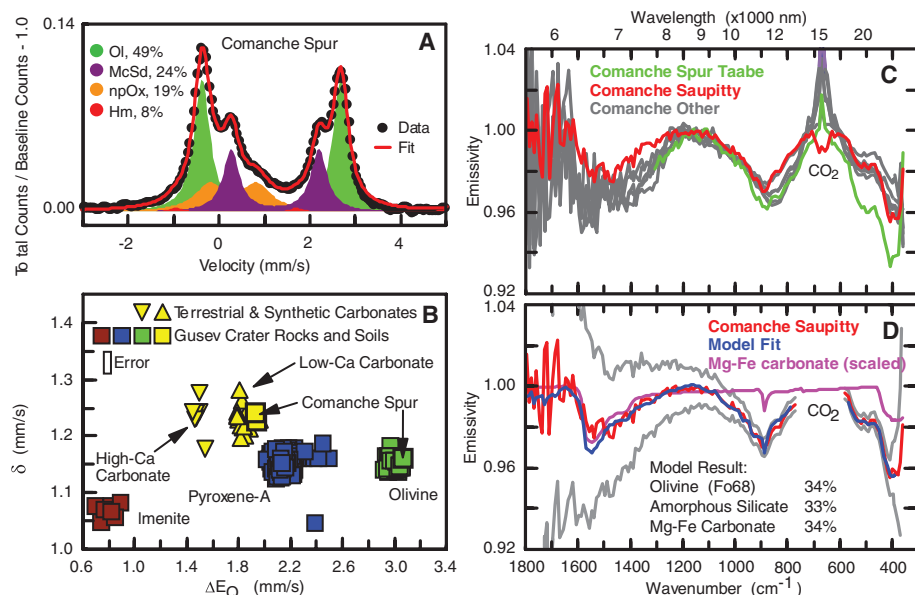


Fig. 3. MB spectra and Mini-TES spectra for Comanche outcrops. **(A)** Comanche Spur MB spectrum [composite of Horseback and Palomino targets (Fig. 2)]. Ol = Fe^{2+} in olivine; McSd = Fe^{2+} in Mg-Fe carbonate; npOx = Fe^{3+} in nanophase ferric oxide; and Hm = Fe^{3+} in hematite. **(B)** MB doublet identification diagram showing Gusev crater rocks and soils [after (19)] and terrestrial and synthetic Fe-bearing carbonates (23). The ranges of values of calcite (Cc) for the low- and high-Ca terrestrial and synthetic carbonates are 0.00 to 0.17 mol fraction and 0.47 to 0.95 mol fraction, respectively. The uncertainty of the subspectral area is $\pm 2\%$ absolute. Center shift (δ) = $(v_2 - v_1)/2$ and $\Delta E_Q = v_2 - v_1$, where v_1 and v_2 are peak center positions numbered from low to high velocity, and ΔE_Q is the quadrupole splitting. **(C)** Mini-TES spectra for target Taabe on Comanche Spur, target Saupitty on the adjacent Comanche outcrop, and six other targets on Comanche outcrops (Fig. 2). **(D)** Model fit (blue line) for Comanche Saupitty using the three geologic phases shown (23). The laboratory spectrum of Mg-Fe carbonate component is shown for reference. Gray lines are 1 SD of the 200 individual spectra averaged to produce the Saupitty spectrum.

Table 1. Chemical composition of Comanche Spur Palomino whole rock, with light elements as CO_2 and calculated components olivine, carbonate, and residue.

	Whole rock* (wt %)	Component†		
		Olivine‡ (wt %)	Carbonate§ (wt %)	Residue (wt %)
SiO_2	36.1 ± 0.4	37.8	—	62.1
TiO_2	0.22 ± 0.06	—	—	0.66
Al_2O_3	2.56 ± 0.08	—	—	7.68
Cr_2O_3	0.63 ± 0.03	—	—	1.88
Fe_2O_3	4.84 ± 0.03	—	—	14.5
FeO	15.4 ± 0.1	25.6	19.2	—
MnO	0.37 ± 0.01	—	1.43	—
MgO	21.6 ± 0.2	36.4	26.2	—
CaO	1.69 ± 0.02	—	6.55	—
Na_2O	1.0 ± 0.2	—	—	3.0
K_2O	0.03 ± 0.05	—	—	0.1
P_2O_5	0.39 ± 0.07	—	—	1.16
SO_3	2.36 ± 0.04	—	—	7.08
Cl	0.53 ± 0.01	—	—	1.61
CO_2	12 ± 5	—	46.4	—
Chemistry total	99.8	99.8	99.8	99.8
Component total	99.8	40.9	25.7	33.2

*APXS data from (21) recalculated to 12 wt % CO_2 . †All Ca and all Mn calculated as carbonate. ‡Equivalent to $(\text{Mg}_{0.72}\text{Fe}_{0.28})\text{SiO}_4$. §Equivalent to $(\text{Mg}_{0.62}\text{Fe}_{0.25}\text{Ca}_{0.11}\text{Mn}_{0.02})\text{CO}_3$.

whose centers vary from spectrum to spectrum (2.29 to 2.31 μm and 2.49 to 2.52 μm) (6). Using laboratory spectra (31) and an assumed linear relationship in band position between $\text{Mc}_{1.00}$ and $\text{Sd}_{1.00}$, we calculated band centers at 2.31 and 2.51 μm for a Comanche-like carbonate ($\text{Mc}_{0.75}\text{Sd}_{0.25}$). Thus, Comanche and Nili Fossae carbonates have the same composition within VNIR constraints. Like Comanche carbonate, Nili Fossae carbonate is associated with olivine (6, 32, 33).

The apparently similar chemical compositions of Comanche, ALH 84001, and Nili Fossae carbonates suggest a common formation pathway. Although we do not exclude an igneous origin (34), multiple lines of evidence point to aqueous processes under probable hydrothermal conditions. Synthetic carbonate globules with average chemical compositions similar to those of ALH 84001 and Comanche carbonates and MB parameters similar to those of the Comanche carbonate [Fig. 3B and (23)] were produced in the laboratory by precipitation from Mg-rich supersaturated carbonate solutions under hydrothermal conditions ($\sim 150^\circ\text{C}$) and near-neutral pH (~ 6 to 7) (35). Mg-rich carbonate at Spitsbergen (Norway) is interpreted to result from precipitation from hydrothermal solutions in association with volcanic activity (36, 37). Morphologic (layered clastic deposits) and mineralogical (high- SiO_2 and sulfate-rich deposits) manifestations of volcanic activity and hydrothermal processes abound in the vicinity of the Comanche outcrops, especially near Home Plate (17–21).

The inferred styles of hydrothermal activity at the Comanche outcrops and at Home Plate are quite different, with the former dominated by near-neutral pH, carbonate-bearing solutions [by analogy with (35)] and the latter dominated by acid-sulfate solutions (18, 19). If the similarity in chemical composition, mineralogical composition, and association with ultramafic rock for Comanche and ALH 84001 carbonates extends to a common formation era, both carbonates are Noachian in age after ALH 84001 (38). In any case, Comanche outcrops on contemporary Mars may be remnants of a larger carbonate-bearing deposit that evolved in ultramafic rock in association with alkaline-neutral volcanic activity during the Noachian and then preferentially eroded (compared to massive olivine-pyroxene outcrops such as Algonquin) by a combination of aeolian abrasion and chemical decomposition from exposure to acid-sulfate vapors and/or fluids associated with volcanic activity at Home Plate. A plausible source for carbonate-bearing solutions is, by analogy with Spitsbergen (39, 40), extension of the hydrothermal system into or through subsurface carbonate-bearing rocks where carbonate dissolution took place.

The Comanche outcrops and their substantial carbonate concentration (16 to 34 wt %) imply extensive aqueous activity under near-neutral pH conditions that would be conducive to habitable environments on early Mars. The well-separated Nili Fossae and Gusev crater carbonate

locations (~6300 km on a great circle) imply that such environments have multiple occurrences in Noachian terrain. The high carbonate concentration in the Comanche outcrops is evidence for climate models (3) involving a CO₂ greenhouse gas on a wet and warm early Mars and subsequent sequestering of at least part of that atmosphere in carbonate minerals.

References and Notes

1. J. L. Gooding, *Icarus* **33**, 483 (1978).
2. J. L. Gooding, in *The Solar System: Observations and Interpretations, Rubey Volume IV*, M. G. Kivelson, Ed. (Prentice Hall, Upper Saddle River, NJ, 1986), pp. 208–229.
3. J. B. Pollack, J. F. Kasting, S. M. Richardson, K. Poliakoff, *Icarus* **71**, 203 (1987).
4. D. C. Catling, *J. Geophys. Res.* **104**, 16453 (1999).
5. J. C. Bridges *et al.*, *Space Sci. Rev.* **96**, 365 (2001).
6. B. L. Ehlmann *et al.*, *Science* **322**, 1828 (2008).
7. J. L. Bandfield, T. D. Glotch, P. R. Christensen, *Science* **301**, 1084 (2003).
8. M. D. Lane, M. D. Dyar, J. L. Bishop, *Geophys. Res. Lett.* **31**, L19702 (2004).
9. V. E. Hamilton, H. Y. McSweeney Jr., B. Hapke, *J. Geophys. Res.* **110**, E12006 (2005).
10. W. V. Boynton *et al.*, *Science* **325**, 61 (2009).
11. M. P. Golombek *et al.*, *J. Geophys. Res.* **111**, (E2), E02507 (2006).
12. R. V. Morris *et al.*, *J. Geophys. Res.* **111**, E02513 (2006).
13. S. W. Squyres *et al.*, *J. Geophys. Res.* **111**, E02511 (2006).
14. D. W. Ming *et al.*, *J. Geophys. Res.* **111**, E02512 (2006).
15. T. J. McCoy *et al.*, *J. Geophys. Res.* **113**, E06S03 (2008).
16. R. E. Arvidson *et al.*, *J. Geophys. Res.* **113**, E12S33 (2008).
17. S. W. Squyres *et al.*, *Science* **316**, 738 (2007).
18. S. W. Squyres *et al.*, *Science* **320**, 1063 (2008).
19. R. V. Morris *et al.*, *J. Geophys. Res.* **113**, E12S42 (2008).
20. A. S. Yen *et al.*, *J. Geophys. Res.* **113**, E06S10 (2008).
21. D. W. Ming *et al.*, *J. Geophys. Res.* **113**, E12S39 (2008).
22. G. Klingelhöfer *et al.*, *J. Geophys. Res.* **108**, 8067 (2003).
23. Supporting material available on Science Online includes laboratory studies, spectral unmixing of Mini-TES spectra, and Pancam multispectral spectroscopy.
24. R. Rieder *et al.*, *J. Geophys. Res.* **108**, 8066 (2003).
25. J. L. Campbell *et al.*, *J. Geophys. Res.* **113**, E06S11 (2008).
26. P. R. Christensen *et al.*, *J. Geophys. Res.* **108**, 8064 (2003).
27. M. S. Ramsey, P. R. Christensen, *J. Geophys. Res.* **103**, 577 (1998).
28. D. W. Mittlefehldt, *Meteoritics* **29**, 214 (1994).
29. A. H. Treiman, *Meteoritics* **30**, 294 (1995).
30. R. P. Harvey, H. Y. McSweeney Jr., *Nature* **382**, 49 (1996).
31. S. J. Gaffey, *J. Geophys. Res.* **92**, 1429 (1987).
32. T. M. Hoefen *et al.*, *Science* **302**, 627 (2003).
33. V. E. Hamilton, P. R. Christensen, *Geology* **33**, 433 (2005).
34. T. Usui, H. Y. McSweeney Jr., B. C. Clark III, *J. Geophys. Res.* **113**, E12S44 (2008).
35. D. C. Golden *et al.*, *Meteorit. Planet. Sci.* **35**, 457 (2000).
36. A. H. Treiman *et al.*, *Earth Planet. Sci. Lett.* **204**, 323 (2002).
37. A. Steele *et al.*, *Meteorit. Planet. Sci.* **42**, 1549 (2007).
38. L. E. Borg *et al.*, *Science* **286**, 90 (1999).
39. D. Banks *et al.*, *Geothermics* **28**, 713 (1999).
40. H. E. F. Amundsen, W. L. Griffin, S. Y. O'Reilly, *Tectonophysics* **139**, 169 (1987).
41. R.V.M. and D.W.M. acknowledge the NASA Johnson Space Center and the NASA Mars Exploration Program for support. R.V.M. acknowledges the NASA Ames Astrobiology Institute for support. S.W.R. acknowledges the NASA Mars Data Analysis Program for support. G.K. and I.F. acknowledge support by the German Space Agency DLR under contract 50QM9902. A portion of the research described in this paper was carried out at the Jet Propulsion Laboratory, California Institute of Technology, under a contract with NASA. We thank P. B. Niles for carbonate samples, L. Le for carbonate microprobe analyses, and J. L. Campbell for calculation of excess light-element concentrations from APXS data.

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1189667/DC1
SOM Text
Figs. S1 and S2
Tables S1 and S2
References

16 March 2010; accepted 24 May 2010
Published online 3 June 2010;
10.1126/science.1189667
Include this information when citing this paper.

Ultrahigh Porosity in Metal-Organic Frameworks

Hiroyasu Furukawa,¹ Nakeun Ko,² Yong Bok Go,¹ Naoki Aratani,¹ Sang Beom Choi,² Eunwoo Choi,¹ A. Özgür Yazaydin,³ Randall Q. Snurr,³ Michael O'Keeffe,¹ Jaheon Kim,^{2*} Omar M. Yaghi^{1,4*}

Crystalline solids with extended non-interpenetrating three-dimensional crystal structures were synthesized that support well-defined pores with internal diameters of up to 48 angstroms. The Zn₄O(CO₂)₆ unit was joined with either one or two kinds of organic link, 4,4',4''-[benzene-1,3,5-triyl-tris(ethyne-2,1-diyl)]tribenzoate (BTE), 4,4',44''-[benzene-1,3,5-triyl-tris(benzene-4,1-diyl)]tribenzoate (BBC), 4,4',44''-benzene-1,3,5-triyl-tribenzoate (BTB)/2,6-naphthalenedicarboxylate (NDC), and BTE/biphenyl-4,4'-dicarboxylate (BPDC), to give four metal-organic frameworks (MOFs), MOF-180, -200, -205, and -210, respectively. Members of this series of MOFs show exceptional porosities and gas (hydrogen, methane, and carbon dioxide) uptake capacities. For example, MOF-210 has Brunauer-Emmett-Teller and Langmuir surface areas of 6240 and 10,400 square meters per gram, respectively, and a total carbon dioxide storage capacity of 2870 milligrams per gram. The volume-specific internal surface area of MOF-210 (2060 square meters per cubic centimeter) is equivalent to the outer surface of nanoparticles (3-nanometer cubes) and near the ultimate adsorption limit for solid materials.

One of the most important properties of metal-organic frameworks (MOFs) is their high porosity (fraction of void volume to total volume) and high specific surface area, which has led to many applications concerned with gas storage, separations, and catalysis (1–6). An important consideration in maximizing the uptake of gases within porous MOF crystals is to increase the number of adsorptive sites within a given material. The simplest way to accomplish this is to use slim organic linkers in which the faces and edges of the constituent units (such as phenylene rings) are exposed for gas adsorption (7, 8). Thus in principle, expansion of the

organic links should lead to MOFs with ultrahigh porosity.

However, difficulties arise when targeting such MOFs: (i) Expanded links often yield fragile frameworks (9), and (ii) the large void space within the crystal framework makes it generally susceptible to self-interpenetration (two lattices grow and interpenetrate each other), precluding high porosity (10, 11). In this report, we present four examples of MOFs for which it was possible to overcome the two challenges and to obtain materials with the highest porosity yet achieved. Specifically, the synthesis and crystal structures of the four MOFs (MOF-180, -200, -205, and

-210) are described, three of which show exceptional porosity. In particular, MOF-210 exhibits the highest BET (Brunauer-Emmett-Teller) and Langmuir surface area (6240 and 10,400 m² g⁻¹) and pore volume (3.60 cm³ g⁻¹ and 0.89 cm³ cm⁻³ of MOF crystal) yet reported.

In the pursuit of MOFs with ultrahigh porosity, the octahedral Zn₄O(CO₂)₆ has had a prominent role as a building unit in producing structures exhibiting exceptional porosity (Scheme 1) (7, 8, 12–14). Joining such units by 4,4',44''-benzene-1,3,5-triyl-tribenzoate (BTB) and/or 1,4-benzenedicarboxylate (BDC) linkers produces MOF-5, UMCM-2, and MOF-177 (7, 8, 12–14), which heretofore showed the highest BET surface area and pore volume among MOFs (Table 1). We sought to test the likelihood of reaching higher porosities by expanding the links in MOF-177 and by further exploring the role of mixed links in producing the desired structures. We prepared the expanded forms of MOF-177 from 4,4',4''-[benzene-1,3,5-triyl-tris(ethyne-2,1-diyl)]tribenzoate (BTE) and 4,4',4''-[benzene-1,3,5-triyl-tris(benzene-4,1-diyl)]tribenzoate (BBC) to give MOF-180 and MOF-200, respectively, and used mixed 4,4',4''-benzene-1,3,5-

¹Center for Reticular Chemistry at the California NanoSystems Institute, and Department of Chemistry and Biochemistry, University of California Los Angeles (UCLA), 607 Charles E. Young Drive East, Los Angeles, CA 90095, USA. ²Department of Chemistry, Soongsil University, Seoul 156-743, Korea. ³Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL 60208, USA. ⁴UCLA-Department of Energy (DOE) Institute of Genomics and Proteomics, UCLA, 607 Charles E. Young Drive East, Los Angeles, CA 90095, USA.

*To whom correspondence should be addressed. E-mail: jaheon@ssu.ac.kr (J.K.); yaghi@chem.ucla.edu (O.M.Y.)

triyl-tribenzoate (BTB)/2,6-naphthalenedicarboxylate (NDC) and BTE/biphenyl-4,4'-dicarboxylate (BPDC) links to obtain MOF-205 and 210 (Scheme 1 and Table 1). Here, we present the synthesis and

crystal structures of the four MOFs and report their adsorption of nitrogen (77 K, 1 bar), hydrogen (77 K, 80 bar), and methane and carbon dioxide (298 K, 80 and 55 bar, respectively).

Scheme 1. $\text{Zn}_4\text{O}(\text{CO}_2)_6$ unit (left) is connected with organic linkers (middle) to form MOFs.

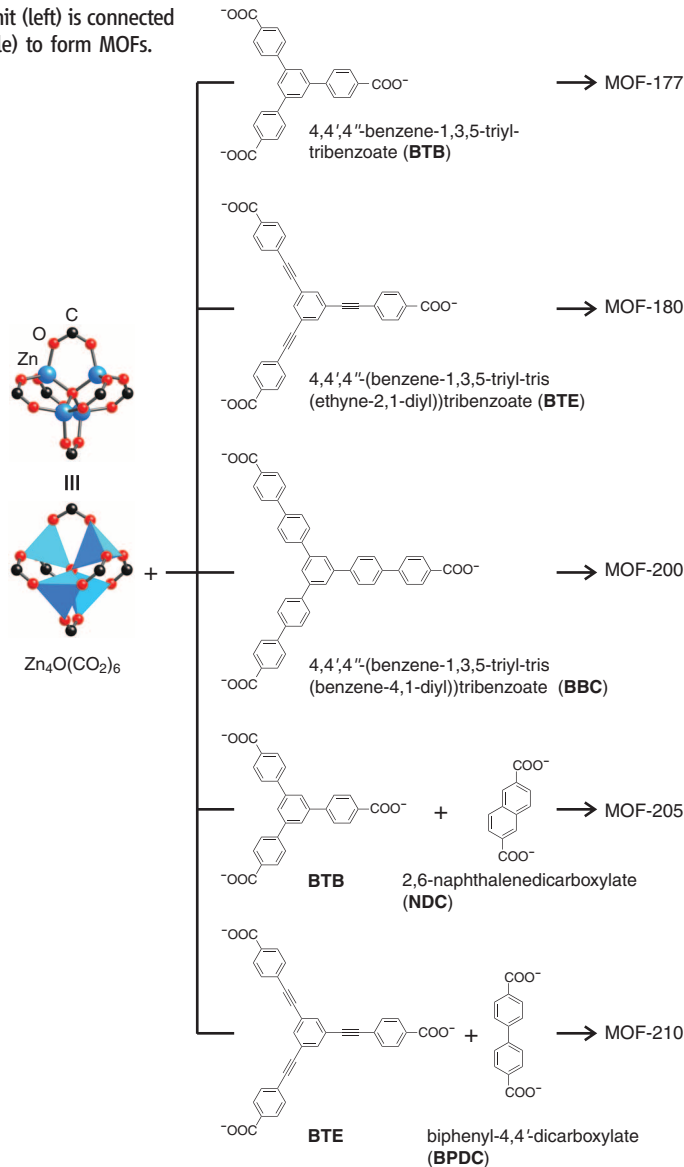


Table 1. Porosity data of highly porous MOFs. A_{BET} , A_{Lang} , and A_{geo} are the BET, Langmuir, and geometric surface areas, respectively. V_p is the measured pore volume. ND, no data; $\text{H}_2\text{T2DC}$, thieno [3,2-*b*]thiophene-2,5-dicarboxylic acid.

Compound	RCSR code	Linker	Void volume (%)	Crystal density (g cm^{-3})	A_{BET} ($\text{m}^2 \text{g}^{-1}$)	A_{Lang} ($\text{m}^2 \text{g}^{-1}$)	A_{geo}^* ($\text{m}^2 \text{g}^{-1}$)	V_p ($\text{cm}^3 \text{g}^{-1}$)	Reference
MOF-5	pcu	BDC	79	0.59	3800	4400	3390	1.55	(14)
MOF-177	qom	BTB	83	0.43	4500	5340	4740	1.89	(22)
MOF-180	qom	BTE	89	0.25	ND	ND	6080	ND	This work
MOF-200	qom	BBC	90	0.22	4530	10400	6400	3.59	This work
MOF-205	ith-d	BTB, NDC	85	0.38	4460	6170	4680	2.16	This work
MOF-210	toz	BTE, BPDC	89	0.25	6240	10400	5850	3.60	This work
UMCM-2	umt	BTB, T2DC	83	0.40	5200	6060	4360	2.32	(13)
MIL-101c	mtm-e	BDC	83	0.44	4230	5900	2880	2.15	(23, 24)

*See section S6 in (17).

It is a basic tenet of reticular chemistry that, in the assembly of variously shaped geometric units, frameworks with highly symmetric vertices and, ideally, one kind of link ("edge transitive") would be most likely to form. In the present case of linking octahedral and triangular units, at first sight the most favorable net ("net" refers to the periodic graph that is the underlying topology of the structure) appears to be **pyr** (15). Indeed, that is the observed net in MOF-150 (16) and related MOFs; however, these form unwanted (denser) structures with two interpenetrating networks. This is unsurprising because **pyr** is a net with a self-dual tiling (the same net from when linkers replace vertices, and vice versa). The interpenetrating dual nets have the same connectivity involving alternating octahedra and triangles (Fig. 1, A and B). We recognized that it is necessary to build a MOF with expanded organic links that is based on a net with a very different from its dual so as to avoid interpenetrating frameworks (Fig. 1, C and D) (7).

In earlier studies, we found that with aromatic tritopic linkers of the sort used here a closely related net (**qom**) is produced in MOF-177 (Fig. 1F) (7), in which alternating octahedral $\text{Zn}_4\text{O}(\text{CO}_2)_6$ and triangular BTB units produce one of the most porous structures yet reported, and for which the interpenetrating dual net involves direct links between octahedral units and between triangular units (Fig. 1, C to E). However, it is impossible to create a MOF with such linkages, and an interpenetrating pair of MOFs does not appear.

Accordingly, an isorecticular non-interpenetrating expansion of MOF-177 was targeted by using BTE or BBC to make the highly porous materials MOF-180 and -200 (Fig. 1, G and H) (17). The unit cell volumes of MOF-180 and -200 are respectively 1.9 and 2.6 times greater than that of MOF-177, with void volumes of 89 and 90% of the crystal volume (Table 1). The cage sizes for MOF-180 and -200 are 15 by 23 and 18 by 28 Å, respectively, which is on the border of micropores and mesopores. The bulk density for MOF-200 is 0.22 g cm^{-3} , implying that the **qom** net is advantageous to reduce the dead space and increase the gas storage capacity per unit volume in a closed tank. This density is the lowest for MOF structures, and of any other crystals at room temperature except for those of the least dense covalent organic frameworks (18).

On the basis of our effective use of the **qom** net for the successful synthesis of the non-interpenetrating MOF-180 and MOF-200, we recognized that other MOFs of nets without self-dual tilings could be made if we used mixed organic links of mixed connectivity. We used both tritopic H_3BTB and ditopic H_2NDC in a reaction with Zn ions to produce $\text{Zn}_4\text{O}(\text{CO}_2)_6$ units and make MOF-205 (Fig. 2A) (17). Its structure belongs to a cubic space group $Pm \bar{3}n$ and consists of one type of $\text{Zn}_4\text{O}(\text{CO}_2)_6$ octahedral unit whose vertices are connected to four BTB and two NDC links [after this work was completed, the same compound was independently reported as DUT-6 (19)]. The topology of MOF-205 (**ith-d**) is of considerable intrinsic interest; all the rings of

the underlying net are 5-rings, and it forms a face-transitive tiling of dodecahedra [5¹²] and tetrahedra [5⁴] in the ratio 1:3 (Fig. 2C). The dual structure (**ith**) is an edge-transitive net with tetrahedral and icosahedral vertices and very different from the original net.

Attempts at isorecticular expansion of MOF-205 by use of the linkers BTE and BPDC produced a different but related material (termed MOF-210) (Fig. 2B). MOF-210 was prepared from a solvothermal reaction of H₃BTE, H₂BPDC, and zinc(II) nitrate hexahydrate (17). Similar to the discovery of MOF-177 (7), this proved to be serendipitous: Rather than the 12-face tile of **ith-d**, the new topology has 30-face tiles, and the full tiling consists of [4⁶.5²⁴], [4³.5⁶], and [5⁴] in the ratio 1:2:3 (Fig. 2D). It is hard to envisage how the connectivity is determined; however, we believe the subtle difference in the link length ratio (for example, 0.76 for MOF-210 and 0.79 for UCMC-2) may be important. The dimension of the largest cage in MOF-210 is 26.9 by 48.3 Å, which comprises 18 Zn₄O units with 14 BTE and 6 BPDC links. The estimated bulk density (void space) is 0.25 g cm⁻³ (89%), which is almost the same as that for MOF-180.

Considering the bulk density and void space calculated from the crystal structure analyses, MOF-200 and -210 are promising candidates to realize ultrahigh surface area. Before gas adsorption measurements, grand canonical Monte Carlo (GCMC) simulations were performed to calculate nitrogen adsorption isotherms (17). Predicted isotherms (Fig. 3A) show unusual steps attributed to the micropore filling at $P/P_0 = 0.12$ and 0.26 (for MOF-200 and -210, respectively), and total nitrogen uptakes in MOF-200 and -210 reaching 2650 and 2300 cm³ g⁻¹, respectively. The BET and Langmuir surface areas determined from these calculated isotherms are respectively 6260 and 12,040 for MOF-200 and 6580 and 10,450 m² g⁻¹ for MOF-210; these are much higher than values reported previously for other porous crystals.

To assess the architectural stability and porosity of these low-density MOFs, and to confirm the calculations, we measured nitrogen adsorption isotherms on the guest free samples of MOF-200, -205, and -210. Preliminary trials revealed that the solvent exchange followed by pore evacuation under vacuum was not effective to activate MOF-200 and -210 without losing the porosity. Thus, these crystals were fully exchanged with liquid CO₂, they were kept under supercritical CO₂ atmosphere, and then their pores were bled of CO₂ in order to yield activated samples (20, 21). Successful guest removal was confirmed by powder x-ray diffraction measurements and elemental analyses (21). As shown in Fig. 3A, all MOF samples show distinctive steps ($P/P_0 = 0.14$, 0.09, and 0.27 for MOF-200, -205, and -210, respectively), and the profiles for MOF-200 and -210 are nearly the same as the predicted isotherms. The maximum nitrogen uptake capacities at 77 K in MOF-200, -205, and -210 are 2340, 1410, and 2330 cm³ g⁻¹, respectively.

These uptake values in MOF-200 and -210 are well beyond those observed for other crystalline porous solids (7, 13, 14, 22–24). Further, the measured values are near the values predicted on the basis of the structure, indicating that these materials are well activated. Because of the successful sample activation, extremely high BET (and Langmuir) surface areas were obtained: 4530 (10,400), 4460 (6,170), and 6240 (10,400) m² g⁻¹ for MOF-200, -205, and -210, respectively (25). The BET surface area of MOF-210 is the highest reported for crystalline materials. It has recently been shown that

the BET method applied to nitrogen adsorption isotherms provides physically meaningful values for the surface areas of MOFs (25).

Given the exceptional properties of such materials, it is expected that MOFs with ultrahigh surface area would exhibit exceptional gas storage capacity. Accordingly, this series of MOFs was subjected to high-pressure hydrogen (77 K) and methane (298 K) adsorption so as to examine their potential utility in the storage of gaseous fuels (Fig. 3, B and C, and table S12). In hydrogen isotherms, these MOFs reach saturation

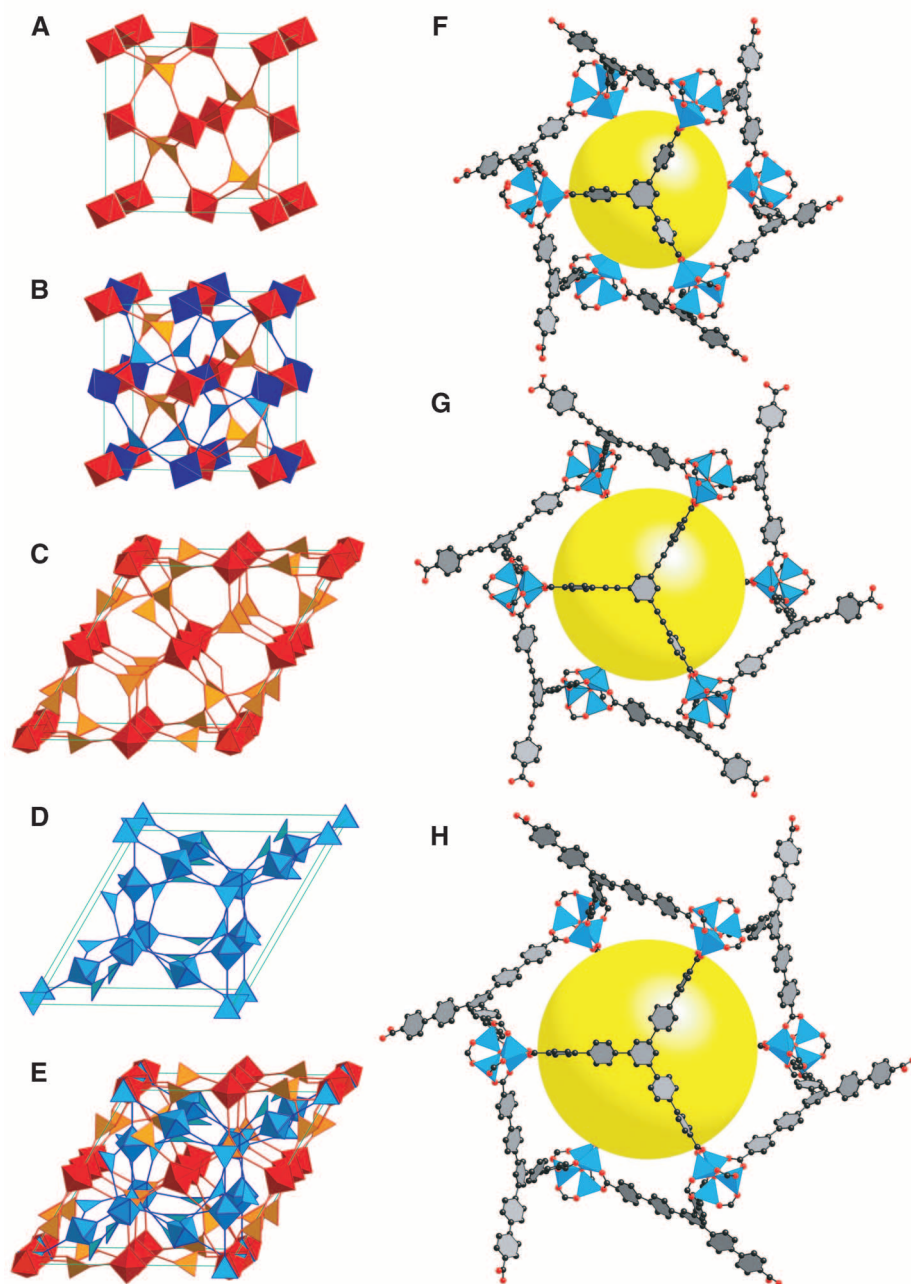


Fig. 1. Connectivity of **pyr** and **qom** (6,3)-coordinated nets. For **pyr** (A), pairs of **pyr** nets can naturally interpenetrate (B). In contrast, **qom** is not self-dual (C to E); the connectivity of the net of the dual tiling for **qom** (D) is very different from the original (C). Crystal structures of MOF-177 (F), MOF-180 (G), and MOF-200 (H) are found in **qom** net (C). The yellow ball is placed in the structure for clarity and to indicate space in the cage. Zn, blue, tetrahedral; O, red; and C, black. Hydrogen atoms are omitted for clarity.

Fig. 2. Crystal structures of MOF-205 (A) and MOF-210 (B). The yellow and orange balls are placed in the structure for clarity and to indicate space in the cage. Atom colors are the same as in Fig. 1. Tiling of (C) **ith-d** and (D) **toz** nets.

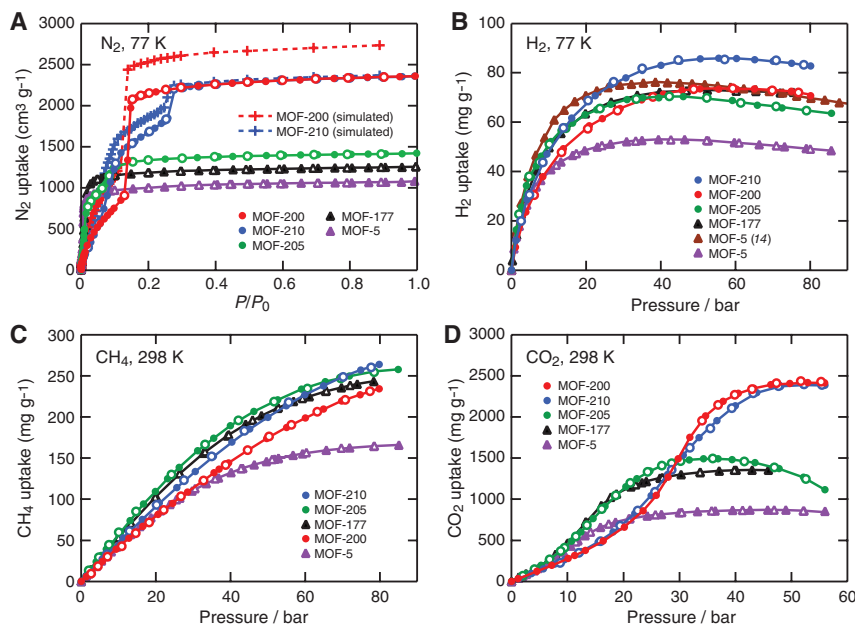
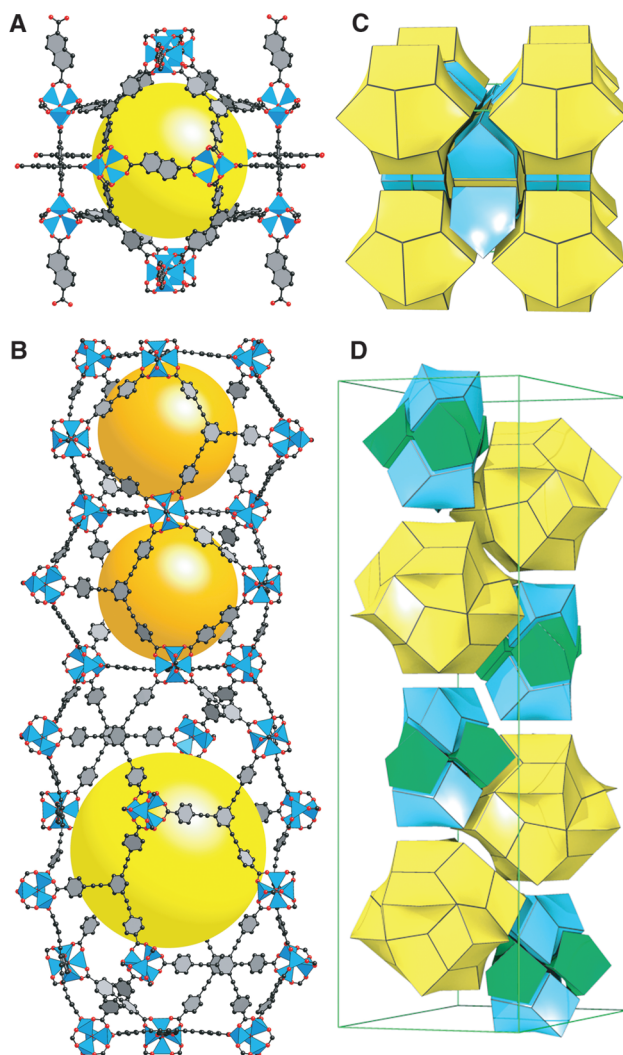


Fig. 3. (A) Low-pressure N_2 isotherms of MOF-5, -177, -200, -205, and -210 at 77 K. Simulated isotherms of MOF-200 and -210 were overlaid. P/P_0 , relative pressure. High-pressure H_2 isotherms were measured at 77 K (B), and (C) CH_4 and (D) CO_2 isotherms were measured at 298 K of the same MOFs.

uptakes, and the saturation pressure increases with an increase in the cavity size. The surface excess hydrogen uptake in MOF-210 (86 mg g^{-1}) is higher than those in MOF-5, MOF-177, UCMC-2, and NOTT-112 (13, 14, 22, 26, 27). The total uptake that a material can store is more relevant to the practicability of using H_2 as a fuel, but it cannot be measured experimentally. Therefore, we estimated this value using the pore volume and the density of hydrogen at 77 K (22). The calculated gravimetric hydrogen density in MOF-210 (176 mg g^{-1}) exceeds that of typical alternative fuels (methanol and ethanol) and hydrocarbons (pentane and hexane). MOF-200 and -205 also show large total hydrogen uptake (163 and 123 mg g^{-1} , respectively); again, these values are higher than MOF-177 (22).

Methane uptake was measured at 298 K and up to 80 bar (Fig. 3C); under the present experimental conditions, all isotherms were not saturated. Although the excess methane uptake in MOF-200, -205, and -210 (234 , 258 , and 264 mg g^{-1} at 80 bar, respectively) were smaller than that in PCN-14 (253 mg g^{-1} at 290 K and 35 bar, respectively) (28), the calculated total uptakes (446 , 394 , and 476 mg g^{-1} for MOF-200, -205, and -210, respectively) were more than 50% greater than those of PCN-14. Moreover, the corresponding volumetric methane densities in the present MOFs are respectively 2, 3, and 2.5 times greater than volumetric bulk density (grams per liter) of methane at the same temperature and pressure (table S12). Because the isotherms are nearly linear up to 80 bar, these materials can deliver most of the sorbed methane in the pressure range between 10 to 200 bar.

Large storage volumes should also be desirable for short-term CO_2 storage. High-pressure CO_2 isotherms for all three MOFs were collected and are presented in Fig. 3D. These MOFs show sigmoidal isotherms, and the pressure for the steep rise reflects the pore size of the MOFs. An isotherm for MOF-205 is saturated at a pressure of 37 bar, whereas the saturation pressure for MOF-200 and -210 are ~ 50 bar. In contrast to hydrogen and methane uptakes, the amounts of excess CO_2 uptake are directly related to the total pore volume. The CO_2 uptake value of 2400 mg g^{-1} in both MOF-200 and -210 exceeds those of any other porous material, such as MOF-177 and MIL-101(Cr) (1470 and 1760 mg g^{-1} , respectively) (24).

The ultrahigh surface areas exhibited by MOF-200 and -210 are near the ultimate limit for solid materials. To appreciate this, it is useful to note that all these compounds have a volume-specific surface area in the range of 1000 to $2000 \text{ m}^2 \text{ cm}^{-3} = 1 \times 10^9$ to $2 \times 10^9 \text{ m}^{-1}$, and for a cube of edge d the external surface area/volume is $6d^2/d^3 = 6/d$. Thus, for a monodisperse powder of cubic nanoparticles to have external surface that is equal to that of these MOFs the cube edge would have to be only 3 to 6 nm, which is a size far too small to practically realize in stable dry powders and therefore impossible to access the full surface area of such particles. This analysis

emphasizes that MOFs are truly “nanomaterials” in the sense that they can be designed to give volume-specific surface areas that are equal to the external surface areas of nanometer-sized particles.

References and Notes

- U. Mueller *et al.*, *J. Mater. Chem.* **16**, 626 (2006).
- S. Kitagawa, R. Kitaura, S. Noro, *Angew. Chem. Int. Ed.* **43**, 2334 (2004).
- J. Y. Lee *et al.*, *Chem. Soc. Rev.* **38**, 1450 (2009).
- X. Zhao *et al.*, *Science* **306**, 1012 (2004).
- M. Dincă, J. R. Long, *Angew. Chem. Int. Ed.* **47**, 6766 (2008).
- O. M. Yaghi *et al.*, *Nature* **423**, 705 (2003).
- H. K. Chae *et al.*, *Nature* **427**, 523 (2004).
- J. L. C. Rowsell, E. C. Spencer, J. Eckert, J. A. K. Howard, O. M. Yaghi, *Science* **309**, 1350 (2005).
- J. K. Schnobrich, K. Koh, K. N. Sura, A. J. Matzger, *Langmuir* **26**, 5808 (2010).
- B. Chen, M. Eddaoudi, S. T. Hyde, M. O’Keeffe, O. M. Yaghi, *Science* **291**, 1021 (2001).
- X. Lin, J. Jia, P. Hubberstey, M. Schröder, N. R. Champness, *CrystEngComm* **9**, 438 (2007).
- H. Li, M. Eddaoudi, M. O’Keeffe, O. M. Yaghi, *Nature* **402**, 276 (1999).
- K. Koh, A. G. Wong-Foy, A. J. Matzger, *J. Am. Chem. Soc.* **131**, 4184 (2009).
- S. S. Kaye, A. Dailly, O. M. Yaghi, J. R. Long, *J. Am. Chem. Soc.* **129**, 14176 (2007).
- M. O’Keeffe, M. A. Peskov, S. J. Ramsden, O. M. Yaghi, *Acc. Chem. Res.* **41**, 1782 (2008).
- H. K. Chae, J. Kim, O. D. Friedrichs, M. O’Keeffe, O. M. Yaghi, *Angew. Chem. Int. Ed.* **42**, 3907 (2003).
- Materials and methods are available as supporting material on Science Online.
- H. M. El-Kaderi *et al.*, *Science* **316**, 268 (2007).
- N. Klein *et al.*, *Angew. Chem. Int. Ed.* **48**, 9954 (2009).
- A. P. Nelson, O. K. Farha, K. L. Mulfort, J. T. Hupp, *J. Am. Chem. Soc.* **131**, 458 (2009).
- C. J. Doonan, W. Morris, H. Furukawa, O. M. Yaghi, *J. Am. Chem. Soc.* **131**, 9492 (2009).
- H. Furukawa, M. A. Miller, O. M. Yaghi, *J. Mater. Chem.* **17**, 3197 (2007).
- G. Férey *et al.*, *Science* **309**, 2040 (2005).
- P. L. Llewellyn *et al.*, *Langmuir* **24**, 7245 (2008).
- K. S. Walton, R. Q. Snurr, *J. Am. Chem. Soc.* **129**, 8552 (2007).
- A. G. Wong-Foy, A. J. Matzger, O. M. Yaghi, *J. Am. Chem. Soc.* **128**, 3494 (2006).
- Y. Yan *et al.*, *Chem. Commun. (Camb.)* **2009**, 1025 (2009).
- S. Ma *et al.*, *J. Am. Chem. Soc.* **130**, 1012 (2008).
- This work is partially supported by BASF SE. The synthesis, characterization of MOF-200, and nitrogen, methane, and carbon dioxide adsorption measurements of the MOFs are supported by the DOE Office of Basic Energy Sciences (DE-FG02-08ER15935 to O.M.Y.), and hydrogen adsorption measurements are supported by the DOE (DE-FG36-05GO15001 to O.M.Y.). We thank the Hydrogen Energy R&D Center, one of the 21st Century Frontier R&D Programs (the Ministry of Education, Science and Technology of Korea to J.K.), and the Defense Threat Reduction Agency (HDTRA1-08-C-005 to R.Q.S.). We thank N. W. Ockwig (Sandia National Laboratories) for his initial work, S. Khan (UCLA) for his help in single-crystal x-ray structure collection and analysis of MOF-200, Y. K. Park and E. Jo (Soongsil University) for their synthesis work of organic links, Accelrys Korea for MS Modeling support, and Pohang Accelerator Laboratory, Korea (2009-2063-12, 2009-2063-18). Crystallographic data for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre under reference numbers CCDC 775690 to 775693. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK).

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1192160/DC1

Materials and Methods

Figs S1 to S39

Tables S1 to S12

References

11 May 2010; accepted 21 June 2010

Published online 1 July 2010;

10.1126/science.1192160

Include this information when citing this paper.

Calcareous Nannoplankton Response to Surface-Water Acidification Around Oceanic Anoxic Event 1a

Elisabetta Erba,^{1*} Cinzia Bottini,¹ Helmut J. Weissert,² Christina E. Keller²

Ocean acidification induced by atmospheric CO₂ may be a major threat to marine ecosystems, particularly to calcareous nannoplankton. We show that, during the Aptian (~120 million years ago) Oceanic Anoxic Event 1a, which resulted from a massive addition of volcanic CO₂, the morphological features of calcareous nannofossils traced the biological response to acidified surface waters. We observe the demise of heavily calcified nannoconids and reduced calcite paleofluxes at the beginning of a pre-anoxia calcification crisis. Ephemeral coccolith dwarfism and malformation represent species-specific adjustments to survive lower pH, whereas later, abundance peaks indicate intermittent alkalinity recovery. Deepwater acidification occurred with a delay of 25,000 to 30,000 years. After the dissolution climax, nannoplankton and carbonate recovery developed over ~160,000 years under persisting global dysoxia-anoxia.

The dissolution of an atmospheric CO₂ surplus [that is, over 500 parts per million (ppm)] in the ocean lowers pH and reduces the CaCO₃ saturation state, consequently accelerating carbonate dissolution in the deep sea (1). The effect of modern surface-water acidification on organisms with CaCO₃-based skeletons or tests, such as calcareous nannoplankton, remains elusive (2–6). Throughout Earth’s history, there is evidence of large CO₂ releases, greenhouse conditions, ocean acidification, and major changes in biota, particularly in marine calcifiers (7). In many cases, the geological record indicates that ocean biota can

adapt to increased acidity; however, past examples of ocean acidification occurred over tens of thousands of years, giving time for life to adjust to CO₂ concentrations as high as 2000 to 3000 ppm (7).

The early Aptian [121 to 118 million years ago (Ma)] represents a case history of excess CO₂ derived from a major volcanic episode, namely the emplacement of the Ontong Java Plateau (OJP) (8, 9), which is marked by changes in the evolutionary rates, species richness, abundance, and calcite production of calcareous nannoplankton (10–12). These changes occurred during Oceanic Anoxic Event 1a (OAE1a) (~120 Ma), which was a time of severe global warming (13, 14). Although global anoxia and enhanced organic matter burial are the most striking and intriguing paleoceanographic phenomena during this event, OAE1a sediments reveal a sequence of CO₂ pulses (15) and weathering changes (16). For example, the cutoff of carbonates during

OAE1a is the result of volcanogenic CO₂-related ocean acidification (7, 10, 17).

We analyzed calcareous nannofossil assemblages from two drill sites in the Tethys (Cismon core) and Pacific [Deep Sea Drilling Project (DSDP) site 463] Oceans (fig. S1) (18). At both sites, nannofossil changes integrated with geochemical and cyclochronological data (15, 19) identify and date the effects of acidification on calcareous nannoplankton. Shortly before magnetic chron M0 (Fig. 1), at 121.3 Ma (19), nannoconid abundance declined and nannofossil paleofluxes (tracing nannoplankton carbonate production and accumulation) decreased as response to a major injection of volcanogenic CO₂. Later, a sharp nannoconid crisis at 120.25 Ma was part of a global calcification failure of planktonic and benthic calcifiers in pelagic and neritic settings under excess CO₂ in the ocean-atmosphere system (17). During the 1-million-year-long interval between these two events, the geological record reveals subtle effects of ocean acidification traced only by nannofossils, and specifically by the heavily calcified nannoconids, with trivial effects on other coccoliths and apparently no evidence in the lithologic and geochemical records. Although the negative carbon isotopic event (CIE) at the beginning of global anoxia (~120 Ma) coincides with the drop in carbonate content, there was an increase in relative abundance of *Biscutum constans*, *Zeughrabdotus erectus*, and *Discorhabdus rotatorius*, represented by dwarfed specimens (Fig. 1). Size variation was species-specific at both sites, because *B. constans* displays the most pronounced morphometric decrease (a volume/mass reduction of 50 to 60% for single coccoliths), whereas *Z. erectus* diminishes in size to a lesser extent (a volume/mass reduction of 30 to 40% for single coccoliths). *D. rotatorius* also exhibits smaller-than-normal sizes throughout the

¹Dipartimento di Scienze della Terra “Ardito Desio,” Università degli Studi di Milano, via Mangiagalli 34, 20133 Milano, Italy.

²Department of Earth Sciences, Geology, Eidgenössische Technische Hochschule (ETH)–Zentrum, Sonneggstrasse 5, CH-8092 Zürich, Switzerland.

*To whom correspondence should be addressed. E-mail: elisabetta.erba@unimi.it

studied interval, reaching minimum dimensions in the CIE (a volume/mass reduction of 5 to 10% for single coccoliths in the study interval, but up to 70% with respect to the holotype). *Watznaueria barnesiae* shows minor changes in abundance and average size, but several malformed/deformed specimens occur in the CIE interval (fig. S2).

In the Cismon core, approximately 25 to 30 thousand years (ky) after the nannoconid crisis

and just before the first OAE1a black shale, nannofossil assemblages in pseudonodular limestone show a relative increase in abundance and diversity, a minimum of nannoconids, and increased relative abundance of *B. constans*, *Z. erectus*, and *D. rotatorius* (Fig. 2, interval 2). The $\delta^{13}\text{C}$ curve is stable through this interval, and therefore an individual volcanogenic CO_2 pulse is unlikely. We suggest that this pseudonodular limestone is evi-

dence of intense dissolution and reflects a shallowing of the calcite lysocline up to ~1200 m of paleowater depth in the Tethys Ocean, indicating a delayed effect of acidification on deep waters and position of the calcite compensation depth (CCD).

The beginning of global anoxia was likely caused by a paroxysmal volcanic event, evidenced by a decrease of the $\delta^{13}\text{C}$ curve and carbonate

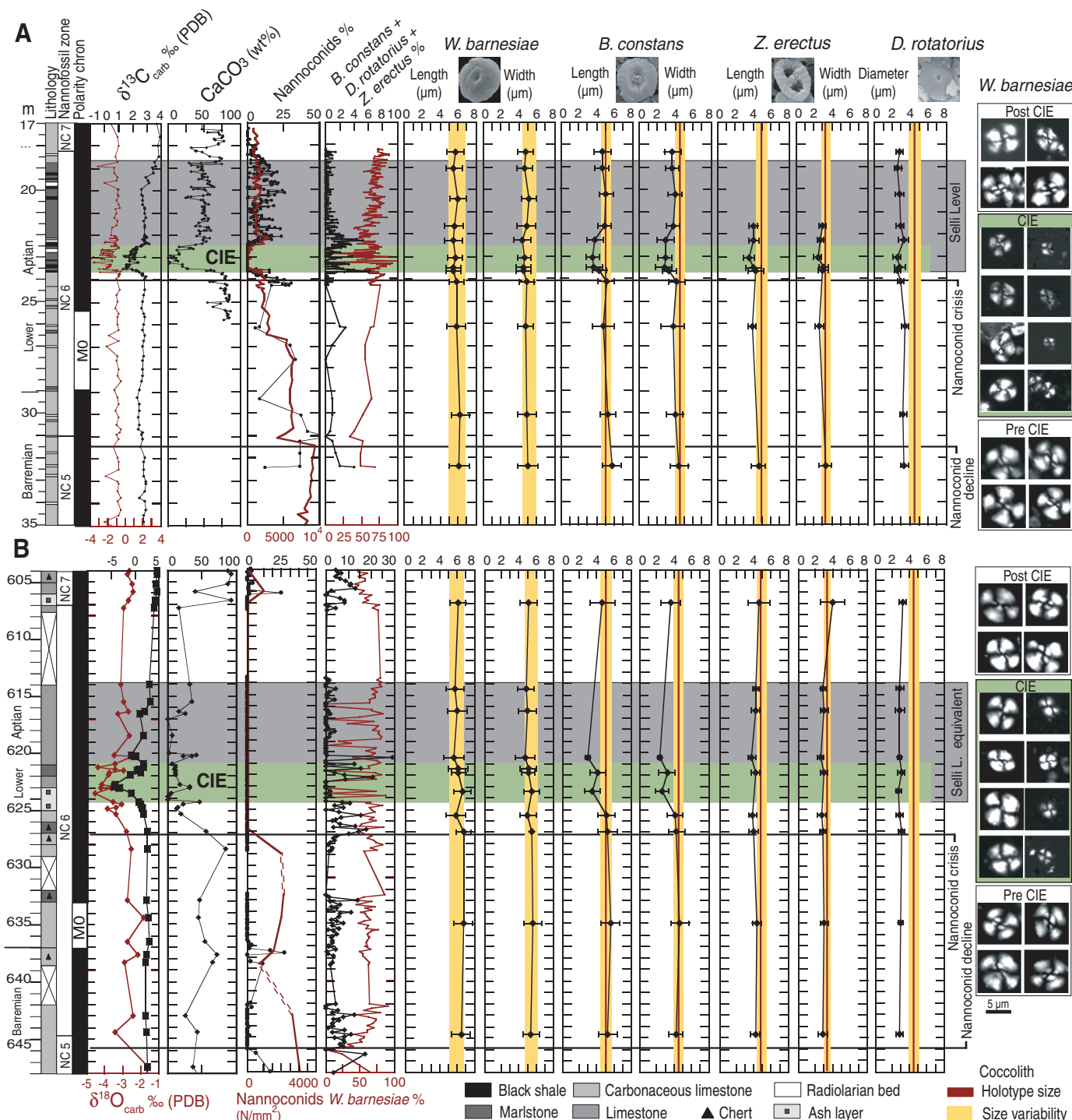


Fig. 1. Average coccolith size ($\pm 1\sigma$) of *W. barnesiae*, *B. constans*, *Z. erectus*, and *D. rotatorius* in the Cismon core (A) and DSDP site 463 (B). Nannofossil abundances are plotted against litho-, bio-, magneto-, and chemostratigraphy

available for both the Cismon core (15, 19, 29) and DSDP site 463 (20, 30). Photographs of *W. barnesiae* document coccolith variations in the pre-CIE, CIE, and post-CIE intervals.

content. Nannofossils did not experience extinction, and insignificant changes in nannofloral abundance and diversity occur simultaneously with the beginning of dwarfism in *B. constans*, *Z. erectus*, and *D. rotatorius* (Fig. 2, interval 3). The smallest coccoliths are not systematically in samples with their highest relative abundance before and after CIE (Fig. 1); therefore, we presume a specific cause for the secretion of dwarf coccoliths. A negative $\delta^{18}\text{O}$ shift that marks interval 3 (lasting <10 ky) suggests a major warming event and is consistent with a coeval episode reconstructed at DSDP site 463 (20). Nannofossil paleofluxes show a progressive decrease at this time, reaching values close to zero in the CIE core (Figs. 2 and 3). Nannofossil paleofluxes do not fully mirror total abundance; heavily calcified taxa, in particular nannoconids, dominate *W. barnesiae* in terms of mass. Likewise, *B. constans*, *Z. erectus*, and *D. rotatorius* increase in abundance, but their coccoliths are too small to affect or compensate for losses in nannofossil calcite paleofluxes.

Shortly after the beginning of OAE1a, in an interval of non-anoxic sediments, a temporary increase in nannoconid abundance and nannofossil paleofluxes, corroborated by a CaCO_3 increase (Fig. 2, interval 4), indicates a ~15-ky-long stasis in the CO_2 input. Indeed, this interval corresponds to less-negative $\delta^{18}\text{O}$ values, suggesting a relative cooling interrupting the initial warming episode. Apparent discrepancy relative to a previous reconstruction (15), which implies a warming pulse derived from biomarkers [interval II of (15)], is possibly caused by different sampling resolution.

Although there is one temperature anomaly at a slightly higher level within the CIE at DSDP site 463 (20), the $\delta^{18}\text{O}$ data show a first warm peak followed by less-negative values at the same stratigraphic levels depicted in the Cismon core.

Another CO_2 pulse [interval III of (15)] corresponds to further reduction of nannoconids and nannofossil paleofluxes and a decrease in carbonate content, $\delta^{13}\text{C}$, and $\delta^{18}\text{O}$ values (interval 5, ~5 ky). Methane hydrate destabilization may be the cause for the most negative $\delta^{13}\text{C}$ values measured in carbonates [interval IV of (15)]; here, nannofossil assemblages remain rare and characterized by dwarf coccoliths, whereas nannoconids show a final exclusion (interval 6, ~5 ky). In this interval, although rare, the heavily calcified *Assipetra* and *Rucinolithus* nannoliths maintain nannofossil paleofluxes above zero (Fig. 3).

In the following ~45-ky-long interval 7 (Figs. 2 and 3), nannofossil assemblages are stable, whereas $\delta^{18}\text{O}$ values gradually increase. The next change in nannofossils suggests strong acidification and dissolution, because dwarf coccoliths exist in the middle part of CIE where nannofossil abundance, diversity, and paleofluxes are least, and CaCO_3 is close to zero. The dissolution-acidification climax (intervals 8 to 10) protracted for ~70 ky, although minor fluctuations occurred. Many samples are barren of nannofossils or exclusively contain rare dissolution-resistant *W. barnesiae*, *Assipetra*, and *Rucinolithus* (mainly in interval 9).

At the beginning of interval 8, the rapid drop in nannofossil abundance, diversity, and paleo-

fluxes, which are substantiated by the short-lived decrease of $\delta^{18}\text{O}$ values, suggests another CO_2 pulse rather than a progressive deterioration of environmental conditions. A minimal increase in CaCO_3 and nannofossil abundance and fluxes, associated with dwarf coccoliths of *B. constans*, *Z. erectus*, and *D. rotatorius*, might represent an acidification pause, perhaps under cooler conditions as suggested by less-negative $\delta^{18}\text{O}$ values (interval 9). Indeed, the coeval increase in relative and absolute abundance of *Assipetra* and *Rucinolithus* (Fig. 3) may suggest a temporary ~35-ky-long slight alkalinity recovery. A further episode of strong dissolution (interval 10, ~20 ky), almost identical to interval 8, is presumably initiated by another CO_2 pulse, as inferred from $\delta^{18}\text{O}$ and small $\delta^{13}\text{C}$ decreases.

The initial recovery (Fig. 3, interval 11) corresponds to a minor increase in nannofossil abundance, paleofluxes, and CaCO_3 during a ~75-ky-long time interval, followed by a more pronounced return to pre-CIE conditions, becoming again suitable for nannoconids (Fig. 3, interval 12). Dwarfism is absent after the full recovery of nannofossil abundance and paleofluxes, mirroring a drop in *B. constans*, *Z. erectus*, and *D. rotatorius* relative abundances (Fig. 1).

Changes in nannofossil assemblages allow the discrimination of surface-water acidification from bottom-water acidity, which postdates the early effects recorded by calcareous nannoplankton. Evidence of bottom-water acidification at 120.22 Ma suggests severe dissolution at the sediment/

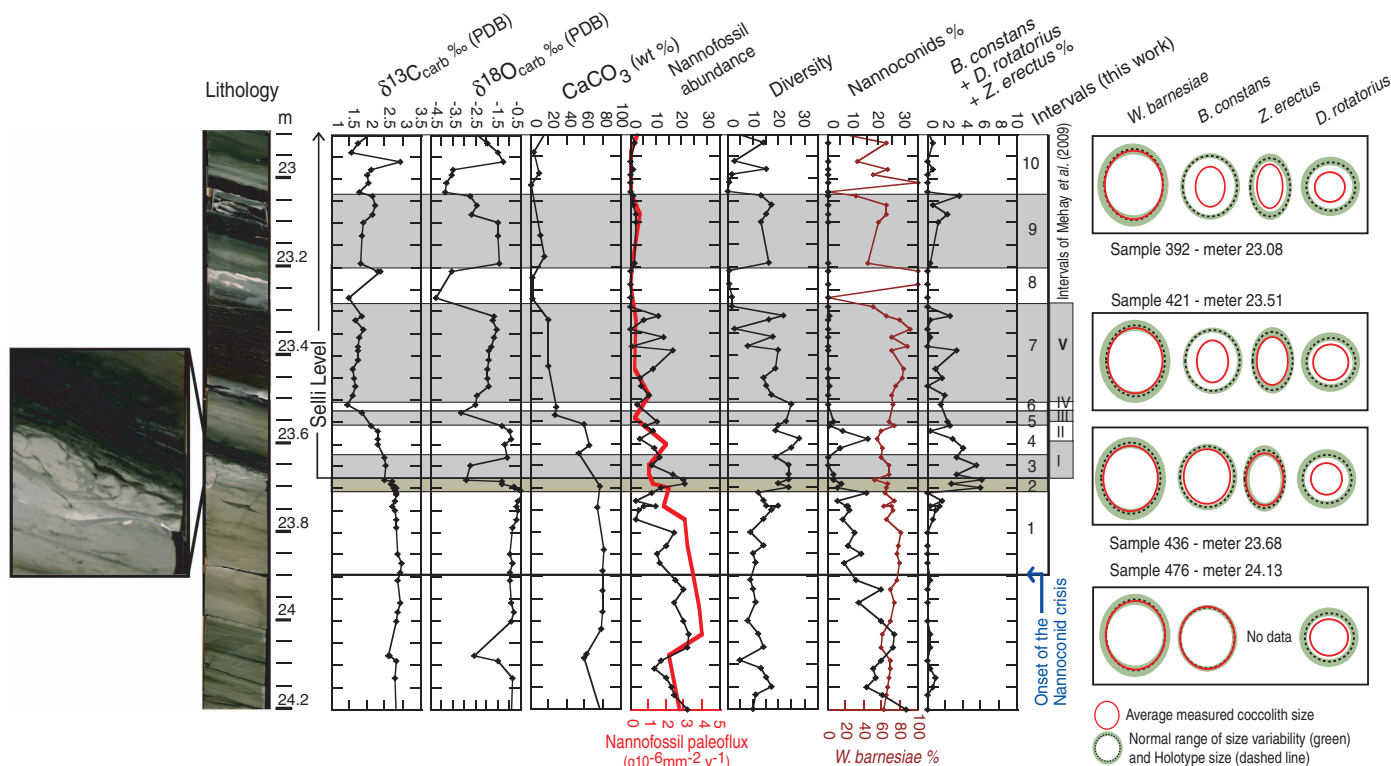


Fig. 2. Close-up of the onset of the OAE1a in the Cismon core. Lithology is an original photograph of the split core (100% core recovery). Nannofossil total abundance and diversity, nannofossil paleofluxes, single taxon relative abundance, and morphometric data are plotted against CaCO_3 , C, and O stable isotopes.

water interface, presumably induced by the shallowing of the calcite lysocline up to ~1200-m water depth. In the first phase of the CIE, nanofossil and CaCO_3 changes enabled a reconstruction of further progressive shoaling of the calcite lysocline and CCD (Fig. 3). The dissolution climax, matching maximum ocean acidification, was ~75 ky after the beginning of the $\delta^{13}\text{C}$ negative anomaly and persisted for ~70 ky. We calculate that this dissolution maximum resulted from shoaling of the Early Cretaceous CCD (21) up to ~1200 m at a rate of 17 m/ky. Recovery occurred at similar rate as the CCD and calcite lysocline deepened to >1200 m. Although the inferred shoaling and deepening of the CCD and calcite lysocline appear to be symmetric, the early acidification pulse recorded by the nannoconid crisis suggests more rapid shoaling at a rate possibly up to 70 m/ky.

The reconstructed CO₂ pulses (Fig. 3) suggest a stepwise accumulation of CO₂ in the ocean inducing progressive acidification. The possibility

exists for a major acceleration of weathering in the lowermost part of OAE1a (16). Indeed, a weathering-induced drawdown of CO₂ may contribute to abundance peak of *Assipetra* and *Rucinolithus* nannoliths (interval 7), possibly representing alkalinity recovery and cooling.

The occurrence of dwarf coccoliths may be the response to a pressure of CO₂ ($p\text{CO}_2$) above threshold levels for most species, but which actually accelerates photosynthesis and growth in some modern r-strategists (organisms that rapidly adapt to and reproduce in unstable conditions) (22). In this scenario, nanmoconids were less CO₂ tolerant, whereas *B. constans*, *Z. erectus*, and *D. rotatorius* were more tolerant, and *W. barnesiae* was even more adaptable. *B. constans*, *Z. erectus*, and *D. rotatorius* are mesotrophic Cretaceous indices, and their synchronous abundance increase in the Tethys and Pacific Oceans (Fig. 1) suggests a global fertilization event. However, dwarfism is not systematically associated with increased abundance

of these mesotrophic taxa, and during OAE1a, the highest primary productivity was recorded above the CIE. Shallow-water calcifiers also experienced a calcification crisis, which was most pronounced in the Atlantic and Tethys Oceans; it coincided with the “mannoconid crisis” and OAE1a (17, 23), indicating that surface waters were loaded with excess CO_2 . The slow and smooth CaCO_3 recovery after the CIE implies a stasis of OJP emplacement and gradual buffering of ocean acidification or a decrease in volcanogenic CO_2 emissions and consistently higher CO_2 drawdown through Corg burial and/or weathering.

A similar, albeit more pronounced, $\delta^{13}\text{C}$ anomaly occurs ~55.8 Ma at the Paleocene Eocene Thermal Maximum (PETM) and also suggests ocean acidification (24). However, the initial decrease in $\delta^{13}\text{C}$ occurred over a few thousand years for the PETM and over ~30 ky for OAE1a. This discrepancy could reflect different causal mechanisms, namely methane hydrate dissociation for PETM (25) and major volcanogenic CO_2 injections for OAE1a, or condensation and hiatuses for most PETM sections. Indeed, severe dissolution in intervals preceding PETM occurs at several deep and intermediate sites (7, 24, 26). The difference in CIE duration (~100 to 150 ky and ~200 ky for the PETM and OAE1a, respectively) might reflect subsequent pulses accumulating excess CO_2 in the ocean-atmosphere system. The recovery phase is also longer for OAE1a (~160 ky versus ~30 to 80 ky), possibly because the more protracted CIE perturbation required a longer time interval for deep-ocean buffering.

The response of calcareous nannoplankton to ocean acidification in the PETM is unclear, because transient, perhaps malformed, taxa may be of solely local importance (27) or indicative of unsaturated surface waters at global scale (26). Reconstructed rates of originations and extinctions at the onset of PETM indicate modest effects of ocean acidification on evolutionary trends (28). It has also been suggested that a major nannoplankton turnover occurred during OAE1a (11). However, our data demonstrate that rising $p\text{CO}_2$ and surface-ocean acidification during OAE1a triggered false extinctions (a so-called Lazarus effect) among calcareous nannoplankton. Conversely, a major origination episode starts approximately 1 My before global anoxia and persists through OAE1a and associated acidification (10). Increasing $p\text{CO}_2$ triggered coccolith malformation and solicited production of r-strategist taxa, which secreted dwarf coccoliths as a strategy to overcome acidification.

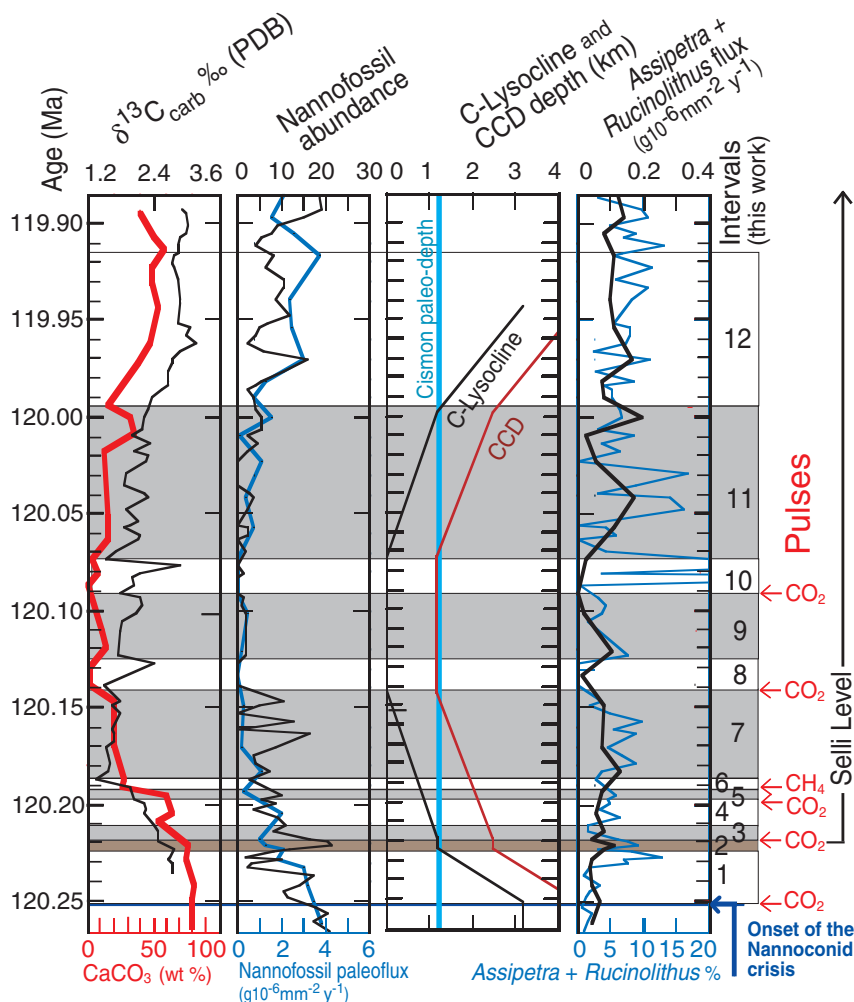


Fig. 3. Details of the CIE interval in the Cismón core. Nannofossil abundance and paleofluxes, relative and absolute abundances of the *Assipetra/Rucinolithus* group, CaCO_3 [weight percent (wt %)], and $\delta^{13}\text{C}$ are plotted versus age (Ma) following the age model of (19). The initial position of the calcite lysocline and CCD are based on the reconstruction by (21) for the Cretaceous Tethys Ocean. The history of the calcite lysocline and CCD reflects the carbonate chemistry evolution in the Tethys Ocean relative to the Cismón paleo-water depth (29).

References and Notes

1. K. Caldeira, M. E. Wickett, *Nature* **425**, 365 (2003).
2. U. Riebesell *et al.*, *Nature* **407**, 364 (2000).
3. M. D. Iglesias-Rodriguez *et al.*, *Science* **320**, 336 (2008).
4. A. Ridgwell *et al.*, *Biogeosciences* **6**, 2611 (2009).
5. J. Barcelos e Ramos, M. N. Müller, U. Riebesell, *Biogeosciences* **7**, 177 (2010).
6. S. Jiang, T. J. Bralower, M. E. Patzkowsky, L. R. Kump, J. D. Schueth, *Nat. Geosci.* **3**, 280 (2010).
7. L. R. Kump, T. J. Bralower, A. Ridgwell, *Oceanography* **22**, 94 (2009).

8. R. L. Larson, *Geology* **19**, 547 (1991).
9. R. L. Larson, E. Erba, *Paleoceanography* **14**, 663 (1999).
10. E. Erba, *Mar. Micropaleontol.* **52**, 85 (2004).
11. R. M. Leckie, T. J. Bralower, R. Cashman, *Paleoceanography* **17**, 1041 (2002).
12. E. Erba, F. Tremolada, *Paleoceanography* **19**, 1008 (2004).
13. A. P. Menegatti *et al.*, *Paleoceanography* **13**, 530 (1998).
14. H. C. Jenkyns, *Philos. Trans. R. Soc. London Ser. A* **361**, 1885 (2003).
15. S. Méhay *et al.*, *Geology* **37**, 819 (2009).
16. M. L. G. Tejada *et al.*, *Geology* **37**, 855 (2009).
17. H. Weissert, E. Erba, *J. Geol. Soc. London* **161**, 695 (2004).
18. Materials and methods are available as supporting material on Science Online.
19. A. Malinverno, E. Erba, T. Herbert, *Paleoceanography* **24**, PA2203 (2010).
20. A. Ando, K. Kaiho, H. Kawahata, T. Kakegawa, *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **260**, 463 (2008).
21. D. Bernoulli, H. C. Jenkyns, *Sedimentology* **56**, 149 (2009).
22. J. B. Ries, A. L. Cohen, D. C. McCorkle, *Geology* **37**, 1131 (2009).
23. M. I. Millán, H. J. Weissert, P. A. Fernández-Mendiola, J. García-Mondéjar, *Earth Planet. Sci. Lett.* **287**, 392 (2009).
24. J. C. Zachos *et al.*, *Science* **308**, 1611 (2005).
25. G. R. Dickens, M. M. Castillo, J. C. G. Walker, *Geology* **25**, 259 (1997).
26. I. Raffi, B. De Bernardi, *Mar. Micropaleontol.* **69**, 119 (2008).
27. P. Bown, P. Pearson, *Mar. Micropaleontol.* **71**, 60 (2009).
28. S. J. Gibbs, P. R. Bown, J. A. Sessa, T. J. Bralower, P. A. Wilson, *Science* **314**, 1770 (2006).
29. E. Erba *et al.*, *J. Foraminiferal Res.* **29**, 371 (1999).
30. E. Erba, *Paleoceanography* **9**, 483 (1994).
31. E.E. and C.B. were funded through MIUR (Italian Ministry of University and Research), Research Programs of National Interest (PRIN) grant 2007-2007W9B2WE 001. H.J.W. and C.E.K. were funded by the Swiss Science Foundation (project 200021-113687) and by ETH Zurich. E.E. and C.B. investigated calcareous nannofossil assemblages, H.J.W. and C.E.K. analyzed C and O isotopes. All authors contributed to the synthesis and paleoceanographic model. Samples from DSDP site 463 were supplied by the Integrated Ocean Drilling Program.

Supporting Online Material

www.sciencemag.org/cgi/content/full/329/5990/428/DC1
Materials and Methods
Figs. S1 and S2
Tables S1 to S3
References

1 March 2010; accepted 18 June 2010
10.1126/science.1188886

The Landscape of *C. elegans* 3'UTRs

Marco Mangone,^{1*} Arun Prasad Manoharan,^{2*} Danielle Thierry-Mieg,^{3*} Jean Thierry-Mieg,^{3*} Ting Han,^{2*} Sebastian D. Mackowiak,⁴ Emily Mis,¹ Charles Zegar,¹ Michelle R. Gutwein,¹ Vishal Khivansara,² Oliver Attie,¹ Kevin Chen,^{1,4} Kourosh Salehi-Ashtiani,^{5,6} Marc Vidal,^{5,6} Timothy T. Harkins,⁷ Pascal Bouffard,⁸ Yutaka Suzuki,⁹ Sumio Sugano,⁹ Yuji Kohara,¹⁰ Nikolaus Rajewsky,⁴ Fabio Piano,^{1,11†} Kristin C. Gunsalus,^{1,11†} John K. Kim^{2†}

Three-prime untranslated regions (3'UTRs) of metazoan messenger RNAs (mRNAs) contain numerous regulatory elements, yet remain largely uncharacterized. Using polyA capture, 3' rapid amplification of complementary DNA (cDNA) ends, full-length cDNAs, and RNA-seq, we defined ~26,000 distinct 3'UTRs in *Caenorhabditis elegans* for ~85% of the 18,328 experimentally supported protein-coding genes and revised ~40% of gene models. Alternative 3'UTR isoforms are frequent, often differentially expressed during development. Average 3'UTR length decreases with animal age. Surprisingly, no polyadenylation signal (PAS) was detected for 13% of polyadenylation sites, predominantly among shorter alternative isoforms. Trans-spliced (versus non-trans-spliced) mRNAs possess longer 3'UTRs and frequently contain no PAS or variant PAS. We identified conserved 3'UTR motifs, isoform-specific predicted microRNA target sites, and polyadenylation of most histone genes. Our data reveal a rich complexity of 3'UTRs, both genome-wide and throughout development.

The 3' untranslated regions (3'UTRs) of mRNAs contain cis-acting sequences that interact with RNA-binding proteins and/or small noncoding RNAs [such as micro RNAs (miRNAs)] to influence mRNA stability, localization, and translational efficiency (1–3). The differential processing of mRNA 3' ends has evident roles in development, metabolism, and disease (4, 5). Despite these critical roles, genome-wide characterization of 3'UTRs lags far behind that of coding sequences (CDSs). Even in the well-annotated genome of *Caenorhabditis elegans*, nearly half (~47%) of the 20,191 genes annotated in WormBase (release WS190) (6, 7) lack an annotated 3'UTR, and only ~1180 (~5%) are annotated with alternative 3'UTR isoforms (fig. S1, A and B).

We have taken a multifaceted, empirical approach to defining the 3'UTR landscape in *C. elegans* (figs. S2 to S5 and tables S1 to S4) (8). We prepared developmentally staged cDNA libraries composed of mostly full-length clones spanning from 5' capped first base to polyadenylated (polyA) tail, and we annotated 16,659 polyA addition sites in 11,180 genes by manually curating ~300,000 Sanger capillary sequence traces in National Center for Biotechnology Information (NCBI) AceView (9). We developed a method to capture the 3' ends

of polyadenylated transcripts genome-wide by deep sampling and generated a comprehensive developmental profile comprising more than 2.5 million sequence reads from Roche/454 (fig. S2 to S5 and tables S1 to S4). We cloned 3' rapid amplification of cDNA ends (RACE) products directly targeting 3'UTRs for 7105 CDSs (6741 genes) in both the Promoterome (10) and ORFeome (11) collections, and we recovered one or more sequenced isoforms for 85% of the targets (figs. S2 and S5 and tables S1 to S4) (8, 12). Finally, we remapped and annotated polyA addition sites in published RNA-seq data (13, 14).

All data sets were mapped, cross-validated, consolidated, and filtered to eliminate obvious experimental artifacts, including internal priming on A-rich stretches (Fig. 1A) (8). These data sets are not yet saturated: Whereas for most genes (11,516 or 73%), at least one 3'UTR isoform is supported by two or more experimental approaches, 47% of transcripts are observed by only one method (in part due to limitations specific to each protocol) (Fig. 1 and tables S3 and S4) (8). The resulting 130,090 distinct polyA sites, identified at single-nucleotide resolution and supported by more than 3 million independent polyA tags, were clustered into 26,967 representative polyA sites. Due to biological varia-

tion, 86% of tags occur within 4 nucleotides of representative sites, although individual polyA tags may spread over ~20 nucleotides (fig. S6).

Linking polyA sites to their parent genes proved to be a challenge, as many previous gene models were incomplete or incompatible with our new data. Using all available empirical evidence, we reannotated in AceView the *C. elegans* gene models (9). Of the 15,683 protein-coding genes with both polyA sites and cDNA support, 57% confirm the structure of WormBase WS190 gene models. The remainder encode different proteins, usually representing different cDNA-supported splice patterns: ~25% share the same stop codon, ~12% use a different stop (hundreds of those correspond to fusions or splits of earlier gene models), and ~6% are not yet annotated in WormBase (supporting data sets S1 and S2).

This integrated collection, herein called the 3'UTRome (fig. S1 and data set S2), provides evidence supporting 3'UTR structures for ~74% of all *C. elegans* protein-coding genes in WormBase WS190, including previously unannotated isoforms for ~7397 genes (fig. S1, A to D). The length distribution of 3'UTRs parallels that in WormBase (fig. S1D), with a mean of 211 nu-

¹Center for Genomics and Systems Biology, Department of Biology, New York University, 1009 Silver Center, New York, NY 10003, USA. ²Life Sciences Institute, Department of Human Genetics, University of Michigan, 210 Washtenaw Avenue, Ann Arbor, MI 48109–2216, USA. ³National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD 20894, USA. ⁴Max Delbrück Centrum für Molekulare Medizin, Robe Rössle-Strasse 10, Berlin-Buch, 13092 Berlin, Germany. ⁵Center for Cancer Systems Biology (CCSB) and Department of Cancer Biology, Dana-Farber Cancer Institute (DFCI), Boston, MA 02115, USA. ⁶Department of Genetics, Harvard Medical School, Boston, MA 02115, USA. ⁷Roche Applied Science, Indianapolis, IN 46250, USA. ⁸454 Life Sciences, Roche Applied Science, Branford, CT 06405, USA. ⁹Department of Medical Genome Sciences, Graduate School of Frontier Sciences, University of Tokyo, 4-6-1 Shirokanedai, Minatoku, Tokyo 108-8639, Japan. ¹⁰Genome Biology Lab Center for Genetic Resource Information, National Institute of Genetics, Mishima 411-8540, Japan. ¹¹New York University, Abu Dhabi, United Arab Emirates.

*These authors contributed equally to this work.

†To whom correspondence should be addressed. E-mail: fp1@nyu.edu (F.P.); kcg1@nyu.edu (K.C.G.); jnkim@umich.edu (J.K.K.)

cleotides (nt) (median = 140 nt). The 3'UTRome matches 61% of WormBase 3'UTRs within ± 10 nt (6714 polyA ends for 6563 genes) and contains thousands of longer or shorter isoforms (fig. S1A). We identified 6177 polyA ends for 4466 genes with no previous 3'UTR annotation and discovered 1490 polyA ends for 1031 genes not yet represented in WormBase (fig. S1A and data sets S1 to S3).

We annotate more than one 3'UTR isoform for 43% of 3'UTRome genes (figs. S1 and S7). Of these, a majority (65%) reflects alternative 3'-end formation at distinct locations in the same terminal exon for proteins using the same stop; the remainder use distinct stops in the same last exon or distinct last exons. Very rarely (79 examples), an intron within the 3'UTR is excised or retained (fig. S8), potentially affecting functional sequence content elements (fig. S8C). Indeed, putative binding sites for miRNAs (this study) or ALG-1 (15) were identified in the variable regions of some of these transcripts. About 2% of genes possess five or more 3'UTR isoforms (Fig. 1A and figs. S1B and S7).

To identify putative cis-acting sequences that may play a role in 3'-end formation, we scanned the 50 nt upstream of the cleavage and polyA addition sites for all possible 5- to 10-mers and assigned the most likely polyadenylation signal (PAS) motif to each 3'UTR using an iterative procedure based on enrichment and centering of the *k*-mers. The canonical PAS motif AAUAAA (seen in 39% of 3' ends) and many variants differing by 1 to 2 nt are detected, with distributions all peaking 19 nt upstream of the polyA site (figs. S9, S10, and table S5) (8). The canonical signal predominates in genes with unique 3'UTRs (57%). However, many high-quality 3'UTRs (3658) lack a detectable PAS motif altogether (Fig. 1, B and C). All PAS variants are embedded within a T-rich region that spikes 5 nt downstream of the PAS motif and extends about 20 nt beyond the cleavage site (Fig. 1D). 3'UTRs with no PAS tend to be T-rich throughout, except for a very A-rich eight-nucleotide region just after the cleavage site (Fig. 1D). Thus, a functional PAS motif with strict sequence specificity appears dispensable for 3'-end formation in *C. elegans*.

Among genes with alternative 3'UTRs, successive polyA sites show a marked asymmetry: The longest isoform prefers a PAS, whereas shorter isoforms more often show no PAS (Fig. 1C and fig. S11). The distance between alternative polyA sites peaks at ~ 40 nt, with resonances at ~ 80 and ~ 140 nt (fig. S11A). This regularity suggests that a physical constraint (possibly queuing transcription complexes) could contribute to cleavage and polyA addition at some upstream sites, which may, therefore, depend less on instructive cues from signal sequences.

Because many *C. elegans* genes undergo trans-splicing of a splice leader (SL) to the 5' end of a nascent transcript (16), we asked whether any properties of transcript 5' and 3' ends correlate (Fig. 2, A and B). About 15% of *C. elegans* genes belong to transcriptional units called operons, each containing two to eight genes that can be cotranscribed, cleaved into separate transcripts, polyadenylated, and trans-spliced with specific leaders (Fig. 2, A and B). The first gene in an operon is trans-spliced only to SL1; downstream genes are usually trans-spliced to 1 of 11 other SLs (SL2 to SL12), although we observed that two-thirds of these genes occasionally become trans-spliced to SL1. The processing of adjacent operon transcript ends (cleavage, polyA addition to the upstream transcript, and SL addition to the downstream transcript) is coupled mechanistically by machinery resembling the cis-splicing apparatus (17). Comparing 3'UTRs within operons, we observe that the "first" (SL1-spliced), "middle" (any gene between first and last), and "last" genes progressively decrease in average length (from 266 to 213 nt), number of 3'UTR isoforms per gene (from 2.64 to 2.51), and frequency of 3'UTRs with no PAS (from 23 to 18% in ~ 1400 sites) (Fig. 2B).

However, only a small fraction (13%) of the 7026 mainly SL1-spliced genes clearly belongs to an operon, and these genes differ notably from non-operon SL1-spliced genes in their usage of the canonical AAUAAA hexamer (22% of 1409 sites versus 32% of 10,879 sites, respectively). Furthermore, we observed the canonical PAS motif much more frequently in non-trans-spliced than in SL-containing transcripts (43% of 5131 sites versus 30% of 14,873 sites) (Fig. 2A). Whereas "standard" non-trans-spliced genes have $\sim 30\%$ more 3'UTR isoforms per gene than "isolated" ones having no neighbor within 2 kb (2.4 versus 1.7), these non-trans-spliced genes are more similar to each other than to trans-spliced genes, because they have shorter and fewer 3'UTR isoforms and higher canonical PAS usage. Thus, trans-splicing within operons appears to enhance (directly or indirectly) the activity of noncanonical PAS sequences upstream, and trans-splicing at the 5' end correlates with distinct properties at the 3' end of the same transcript, independent of 5'-end processing downstream.

Unexpectedly, the 3'UTRome reveals polyadenylated transcripts for nearly all histone genes (fig. S12 and table S6). The major class of replication-dependent histones (H2a, H2b, H3, and H4) is not thought to be polyadenylated in

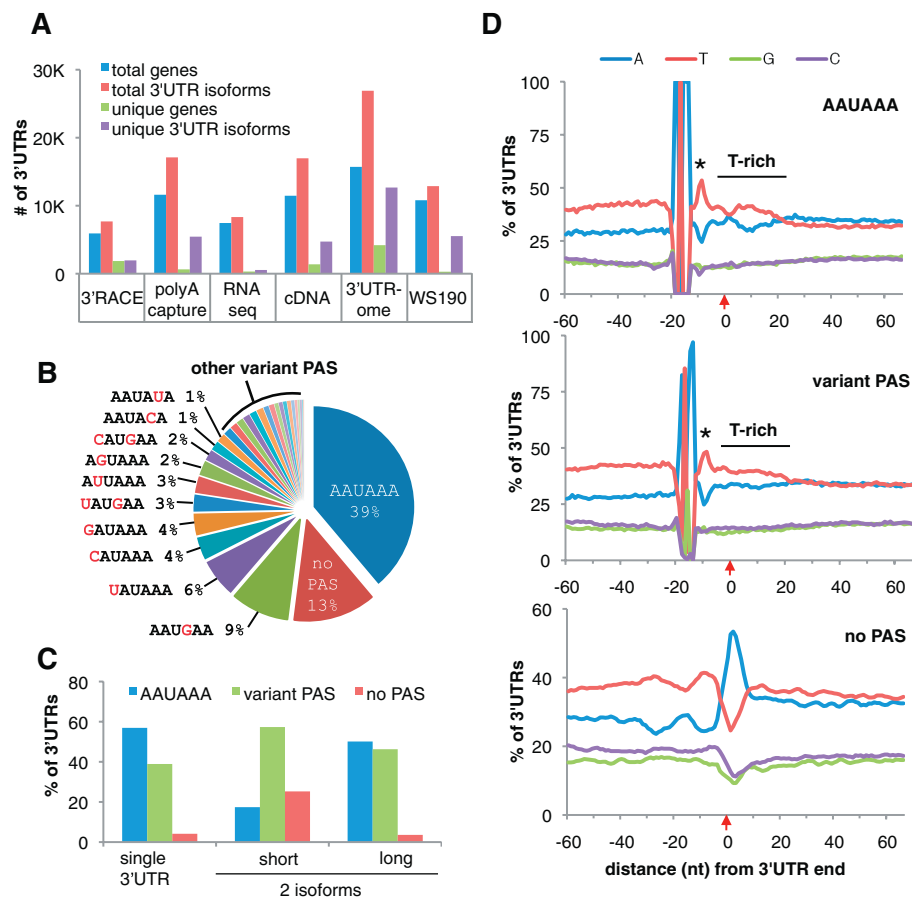


Fig. 1. The 3'UTRome and 3'UTR PAS. **(A)** The number of genes and isoforms detected in, or specific to, each data set and cumulative totals in WS190 and 3'UTRome annotations. **(B)** PAS motif frequencies: AAUAAA (39%), variant PAS (1 to 9%), and no PAS (13%). **(C)** PAS usage in genes with one or two (short and long) 3'UTR isoforms. **(D)** Nucleotide distribution spanning ± 60 nt around the polyA addition site, in 3'UTRs with: AAUAAA (top), 10 most common variant PAS (middle), and no PAS (bottom). Alignments, centered at -19 nt, show a T-spike at 5 nt downstream of PAS (asterisk), polyA addition site (red arrow), and T-rich region downstream of cleavage site. The A-rich peak downstream of "no PAS" is not enriched for AAAAAA, suggesting an A-rich motif at that location rather than artifactual A-rich ends.

metazoans; instead, their 3' ends form a stem-loop structure that is recognized and cleaved several nucleotides downstream by U7 small nuclear ribonucleoprotein and factors such as stem-loop binding protein (18, 19). *C. elegans* has 61 cDNA-supported histone genes (9) that all harbor conserved sequences with 3' stem-loop potential; however, they also contain conserved PAS elements downstream of the hairpin sequence (20). Because *C. elegans* histone transcripts have also been shown to terminate in the typical stem-loop structure and to be depleted in successive rounds of polyA selection (20), we were surprised to recover polyadenylated transcripts for 57 histone genes in multiple, independent data sets (fig. S12 and table S6). This finding suggests that, at least in *C. elegans* (and perhaps also in higher metazoans), the usual route for histone mRNA 3'-end processing may include initial cleavage and polyA addition at conserved PAS sites, followed by further processing to remove sequences downstream of the stem-loop.

We searched 3'UTRs for conserved sequence motifs and other potential functional elements. We updated our atlas of predicted conserved miRNA targets for the 3'UTRome, using the PicTar algorithm with new 3- and 5-way multispecies alignments (Fig. 3, fig. S13, and table S7). Roughly half of the newly predicted sites match our previous predictions (21), but many sites are gained or lost (fig. S13A and table S7). These differences reflect improvements in both 3'UTR annotations and multispecies alignments, which increase the accuracy of conserved-seed site identification and signal-to-noise ratios (8). More than 3000 PAS motifs are positionally conserved among *Caenorhabditis* species, including within alternative 3'UTRs (fig. S13B). Thus, maintenance of multiple specific 3' termini may be functionally important for some genes. Thousands of unexplained conserved sequence blocks of varying lengths within 3'UTRs (Fig. 3B and table S7) may represent previously unrecognized functional elements that await further characterization. In vivo Argonaute (ALG-1) binding sites (15) overlap significantly with predicted miRNA target sites but not with other conserved blocks (table S7), indicating that the latter are, overall, not directly related to microRNA function (8). For 1876 convergently transcribed neighboring genes, overlapping 3' regions could pair as double-stranded RNA if coexpressed, potentially triggering endogenous small interfering RNA production (22) that could down-regulate cognate mRNAs (fig. S14 and data set S4).

We examined alternative 3'UTR isoforms in different developmental stages (Fig. 4) and found a downward trend in average length and number of 3'UTRs per gene from the embryonic through the adult stage (Fig. 4, A and B). Among genes expressed in more than one developmental stage, embryos display the largest proportion of stage-specific 3'UTR isoforms, and these tend toward longer isoforms (Fig. 4, B and C, tables S8 and S9, and data set S5). Some genes switch 3'UTR length coincident with developmental transitions, most notably from embryo to L1, L1 to dauer entry, dauer exit to

L4, and in adult hermaphrodites versus males (Fig. 4D, table S9, and data sets S5 and S6). Thus, 3' UTR-mediated gene regulation may be widespread in the *C. elegans* embryo, and differential expression of alternative isoforms may represent a mechanism to engage or bypass 3'UTR-mediated regulatory controls in specific developmental contexts (23, 24).

The 3'UTRome compendium evidences support for multiple mechanisms of transcript 3'-end formation in *C. elegans*, including standard PAS-directed 3'-end formation from a large collection of PAS variants, regularly spaced "shadow" polyA addition sites devoid of recognizable signals, and both operon-dependent and -independent correlations between features at the 5' and 3' ends of

the same or of consecutive transcripts that are consistent with the possibility that trans-splicing and 3'-end processing within a gene could occur by functionally linked mechanisms. We characterize thousands of previously unknown and alternative 3'UTR isoforms throughout development, define a comprehensive catalog of PAS elements, discover a surprising number of polyadenylated transcripts with no discernable PAS, and definitively document polyadenylation of histone transcripts. We also identify conserved sequence elements in 3'UTRs that may interact with trans-acting factors such as miRNAs and RNA-binding proteins, some of which occur within variable regions of alternative 3'UTRs. A collection of cloned 3'UTRs

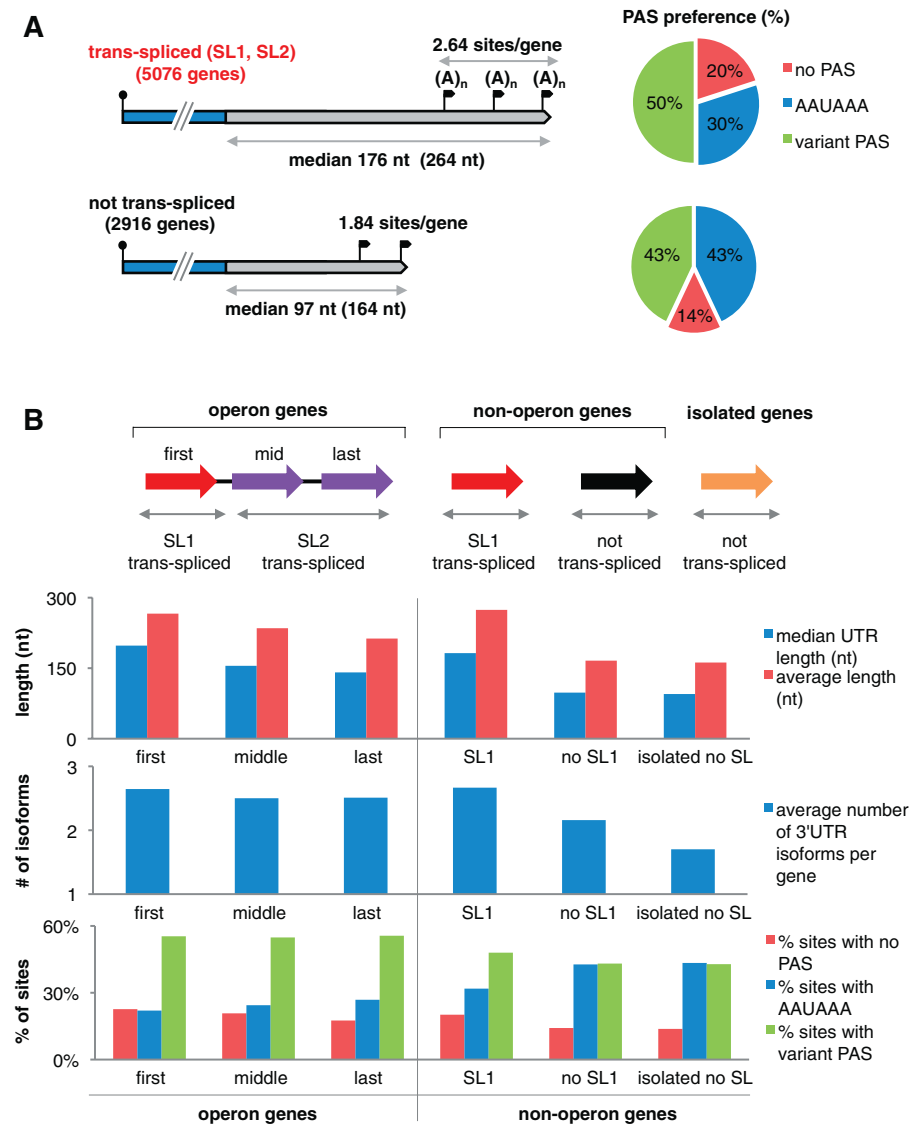


Fig. 2. 3'UTRs in operons and trans-spliced versus non-trans-spliced mRNAs. **(A)** Trans-spliced (top) and non-trans-spliced (bottom) mRNAs: 3'UTR median (and average) lengths, number of 3'UTR isoforms per gene (polyA sites, black flags), and PAS preference (pie charts: % 3'UTRs with AAUAAA, variant PAS, and no PAS). **(B)** (Top) Schematic of operon (left, $n = 574$ operons), non-operon (center, $n = 4348$ genes), and isolated (right, $n = 2098$) genes. Initial operon genes (red) are SL1-trans-spliced; downstream genes (purple) usually acquire one of the other SLs (SL2 to SL12). Non-operon genes are either SL1-trans-spliced (red, $n = 3530$) or not trans-spliced (black, $n = 818$). Isolated genes (having no neighbors within 2 kb) are not trans-spliced (orange, $n = 2098$). (Bottom) 3'UTR lengths, number of isoforms, and PAS sites for operon and non-operon genes.

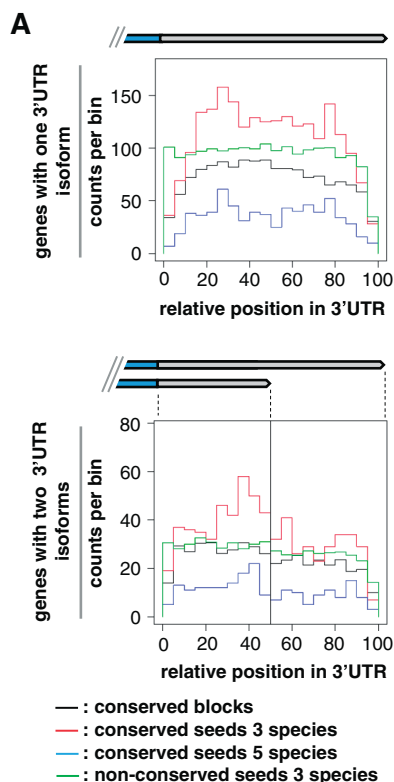


Fig. 3. Conserved sequence elements in 3'UTRs. **(A)** Histogram distributions of conserved sequence blocks (black, counts shown at 1/5th scale), conserved miRNA seeds in three (red; *C. elegans*, *C. remanei*, *C. briggsae*) and five (blue; *C. elegans*, *C. remanei*, *C. briggsae*, *C. brenneri*, *C. japonica*) species, and nonconserved miRNA seeds (green, 1/25th scale) along the normalized length of 3'UTRs, in genes with one isoform (top) or exactly two isoforms (bottom). For genes with one isoform, the length scale is 100%; for two isoforms, 0 to 50% represents the short-isoform span, and 51 to 100% indicates the span exclusive to the long isoform. Counts were binned by fraction of total length and, thus, varied in absolute length. **(B)** Length distribution (up to 20 nt) of conserved sequence blocks in 3'UTRs (excluding miRNA target and PAS sites), in three (blue; $n = 16,204$ conserved blocks) and five (red; $n = 4758$) species. See also table S7.

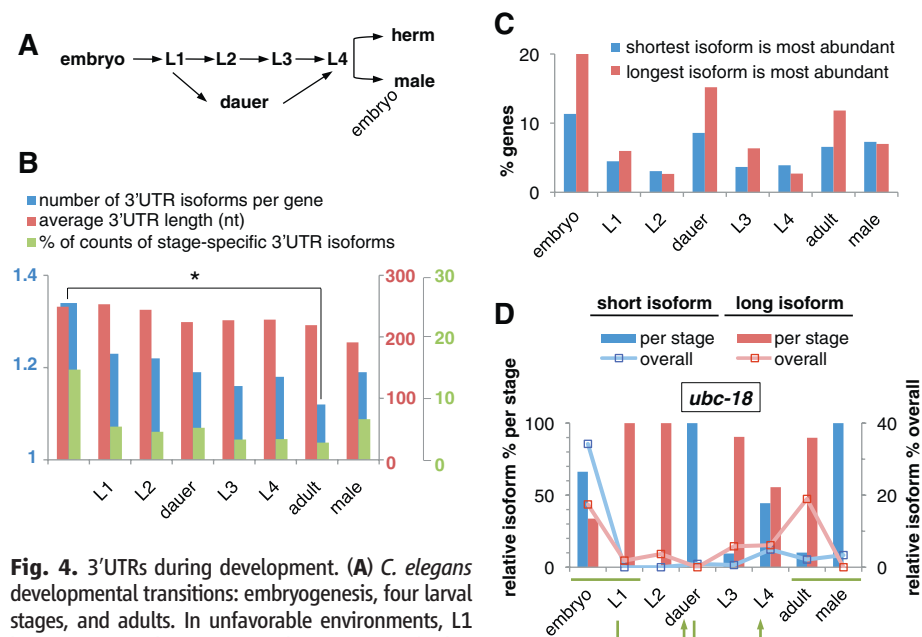


Fig. 4. 3'UTRs during development. **(A)** *C. elegans* developmental transitions: embryogenesis, four larval stages, and adults. In unfavorable environments, L1 larvae arrest in dauer stage and can re-enter the life cycle as L4 larvae. herm, hermaphrodites. **(B)** The number of 3'UTR isoforms per gene decreases significantly during development (blue) (* $p \sim 0.004$, permutation test). The average length of 3'UTRs decreases during development (red). Adult males have shorter average 3'UTRs than hermaphrodites. Embryos show more stage-specific 3'UTR isoforms for genes expressed during multiple developmental stages (green) (see table S8). **(C)** Proportion of genes showing stage-specific expression of alternative 3'UTR isoforms (see table S9). Embryos and dauers favor longer 3'UTR isoforms. **(D)** Differential 3'UTR-isoform expression during development (*ubc-18* shown; see data sets S5 and S6 for details). The bar chart illustrates the relative abundance of short versus long 3'UTR isoforms for *ubc-18* in each stage (sum per stage = 100%, left y axis). The line graph shows the relative abundance across all stages (sum per gene across all stages = 100%, right y axis). Green bars highlight differences in 3'UTR isoform usage in the embryo-to-L1 transition and between adult hermaphrodite and male stages. Green arrows indicate dauer entry and exit transitions.

for several thousand *C. elegans* genes is available to the research community for high-throughput downstream analyses and in vivo studies (table S10 and data set S7) (8).

References and Notes

- C. H. de Moor, H. Meijer, S. Lissenden, *Semin. Cell Dev. Biol.* **16**, 49 (2005).
- M. Wickens, D. S. Bernstein, J. Kimble, R. Parker, *Trends Genet.* **18**, 150 (2002).
- D. P. Bartel, *Cell* **136**, 215 (2009).
- L. He et al., *Nature* **435**, 828 (2005).
- S. Chatterjee, J. K. Pal, *Biol. Cell* **101**, 251 (2009).
- L. Stein, P. Sternberg, R. Durbin, J. Thierry-Mieg, J. Spieth, *Nucleic Acids Res.* **29**, 82 (2001).
- T. W. Harris et al., *Nucleic Acids Res.* **38** (Database issue), D463 (2010).
- See supporting online material for details.
- D. Thierry-Mieg, J. Thierry-Mieg, *Genome Biol.* **7** (suppl. 1), S12.1 (2006).
- D. Dupuy et al., *Genome Res.* **14**, 2169 (2004).
- J. Reboul et al., *Nat. Genet.* **34**, 35 (2003).
- M. Mangone, P. Macmenamin, C. Zegar, F. Piano, K. C. Gunsalus, *Nucleic Acids Res.* **36** (Database issue), D57 (2008).
- L. W. Hillier et al., *Genome Res.* **19**, 657 (2009).
- H. Shin et al., *BMC Biol.* **6**, 30 (2008).
- D. G. Zisoulis et al., *Nat. Struct. Mol. Biol.* **17**, 173 (2010).
- T. Blumenthal et al., *Nature* **417**, 851 (1996).
- Y. Liu, T. Huang, M. MacMorris, T. Blumenthal, *RNA* **7**, 176 (2001).
- Z. F. Wang, M. L. Whitfield, T. C. Ingledue, Z. Dominski, W. F. Marzluff, *Genes Dev.* **10**, 3028 (1996).
- W. F. Marzluff, E. J. Wagner, R. J. Duronio, *Nat. Rev. Genet.* **9**, 843 (2008).
- R. Keall, S. Whitelaw, J. Pettitt, B. Müller, *BMC Mol. Biol.* **8**, 51 (2007).
- S. Lall et al., *Curr. Biol.* **16**, 460 (2006).
- K. Okamura, S. Balla, R. Martin, N. Liu, E. C. Lai, *Nat. Struct. Mol. Biol.* **15**, 581 (2008).
- E. Lund, M. Liu, R. S. Hartley, M. D. Sheets, J. E. Dahlberg, *RNA* **15**, 2351 (2009).
- A. J. Giraldez et al., *Science* **312**, 75 (2006); published online 16 February 2006 (10.1126/science.1122689).
- This work was supported in part by grants from NIH (U01-HG004276) to F.P., K.C.G., J.K.K., and N.R.; NIH grant (R01HG004515) to K.C.; Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to Y.K., S.S., and Y.S.; NIH (R01GM088565), Muscular Dystrophy Association and the Pew Charitable Trusts to J.K.K.; a gift from the Ellison Foundation to M.V. and Institute Sponsored Research funds from the DFCI Strategic Initiative in support of the CCSB; the Helmholtz-Alliance on Systems Biology (Max Delbrück Centrum Systems Biology Network) to S.D.M.; and the Intramural Research Program of NIH, National Library of Medicine to J.T.-M. and D.T.-M. We thank J. V. Moran, T. Blumenthal, A. Billi, D. Mecnas, and B. Bargmann for discussions; T. Shin'i and Exelixis for *C. elegans* cDNA traces; and, for technical assistance, T. Naway and B. Brown (statistical analysis); R. Sachidanandam, R. Lyons, and S. Genik (deep sequencing); P. MacMenamin and D. Schaub (3'UTRome database); M. Morris (data submission); and L. Huang (stage analysis). 3'UTRome data sets are available from NCBI Trace Archive, dbEST, Sequence Read Archive, Gene Expression Omnibus, and modENCODE (8). See supporting online materials and methods for details. Annotations are displayed at NCBI AceView (www.aceview.org) (9) and www.UTRome.org (12).

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1191244/DC1
Materials and Methods
Figs. S1 to S14
Tables S1 to S10
References
Data sets S1 to S7

21 April 2010; accepted 24 May 2010

Published online 3 June 2010;

10.1126/science.1191244

Include this information when citing this paper.

A Septin Diffusion Barrier at the Base of the Primary Cilium Maintains Ciliary Membrane Protein Distribution

Qicong Hu,¹ Ljiljana Milenkovic,^{2,3} Hua Jin,⁴ Matthew P. Scott,^{2,3,5–8} Maxence V. Nachury,⁴ Elias T. Spiliotis,^{9*} W. James Nelson^{1,4*}

In animal cells, the primary cilium transduces extracellular signals through signaling receptors localized in the ciliary membrane, but how these ciliary membrane proteins are retained in the cilium is unknown. We found that ciliary membrane proteins were highly mobile, but their diffusion was impeded at the base of the cilium by a diffusion barrier. Septin 2 (SEPT2), a member of the septin family of guanosine triphosphatases that form a diffusion barrier in budding yeast, localized at the base of the ciliary membrane. SEPT2 depletion resulted in loss of ciliary membrane protein localization and Sonic hedgehog signal transduction, and inhibited ciliogenesis. Thus, SEPT2 is part of a diffusion barrier at the base of the ciliary membrane and is essential for retaining receptor-signaling pathways in the primary cilium.

The primary cilium is an antenna-like organelle protruding from the apical surface of almost every cell in a wide variety of organisms. The ciliary membrane is contiguous with the apical plasma membrane but has a unique set of proteins that sense and transduce a variety of extracellular signals, such as Sonic hedgehog (Shh) (1). These signaling pathways regulate gene expression during development and in adult life,

and mutations in ciliary proteins give rise to a range of developmental defects (2, 3).

Signal transduction by the primary cilium depends on the enrichment of specific proteins in the ciliary membrane. Protein trafficking and intraflagellar transport from the cytoplasm into the cilium establish the characteristic intracellular distributions of ciliary membrane proteins (4, 5), but it is unknown how these

proteins are subsequently retained in the ciliary membrane: For example, they might be immobilized in the cilium or impeded by a diffusion barrier (6–10).

To investigate these possible mechanisms, we used fluorescence recovery after photobleaching (FRAP) (11) to measure the diffusional mobility of four ciliary membrane proteins tagged with green fluorescent protein (GFP): two G protein-coupled receptors [serotonin receptor 6 (^{FI}Htr6^{SEP}) and somatostatin receptor (Sstr3^{GFP}) (12)], and the membrane-anchored and ciliary-targeted cytoplasmic tail (amino acids 1 to 193) of fibrocystin (CTSPKHD1^{GFP}) (13) in primary kidney inner medullary collecting duct (IMCD3)

¹Department of Biology, Stanford University, Stanford, CA 94305, USA. ²Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305, USA. ³Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305, USA. ⁴Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA 94305, USA. ⁵Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305, USA. ⁶Department of Bioengineering, Stanford University, Stanford, CA 94305, USA. ⁷Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305, USA. ⁸Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305, USA. ⁹Department of Biology, Drexel University, Philadelphia, PA 19104, USA.

*To whom correspondence should be addressed. E-mail: wjnelson@stanford.edu (W.J.N.); ets33@drexel.edu (E.T.S.)

Fig. 1. (A) FRAP of proteins in the whole cilium 24 hours after serum starvation. IMCD3 cells stably (a) or transiently (b) expressing Htr6^{SEP} or IFT88^{YFP} (d), and Smo^{YFP} stably expressed in MEFs treated with 100 nM SAG for 24 hours (c); arrowheads mark one end of the cilium. Scale bars, 5 μ m. **(B)** Kinetics of average ($\pm$ SEM) fluorescence recovery of proteins photobleached in the whole cilium (a, b; $n = 8$ to 12). Summary of fluorescence recovery of ciliary membrane proteins as a percentage of the initial unbleached fluorescence level compared with IFT88^{YFP} distribution (c; $***P < 0.0001$). **(C)** FRAP of Htr6^{SEP} (a), Smo^{YFP} (b), and IFT88^{YFP} (c) represented as heat-map images after photobleaching part of the cilium in IMCD3 cells; dotted lines mark the photobleached/unbleached boundary. Scale bars, 5 μ m. **(D)** Representative example of kinetics of fluorescence recovery of photobleached region (orange curve), unbleached region (blue curve), and the two regions combined (red curve) of a primary cilium ($n = 12$ to 13).

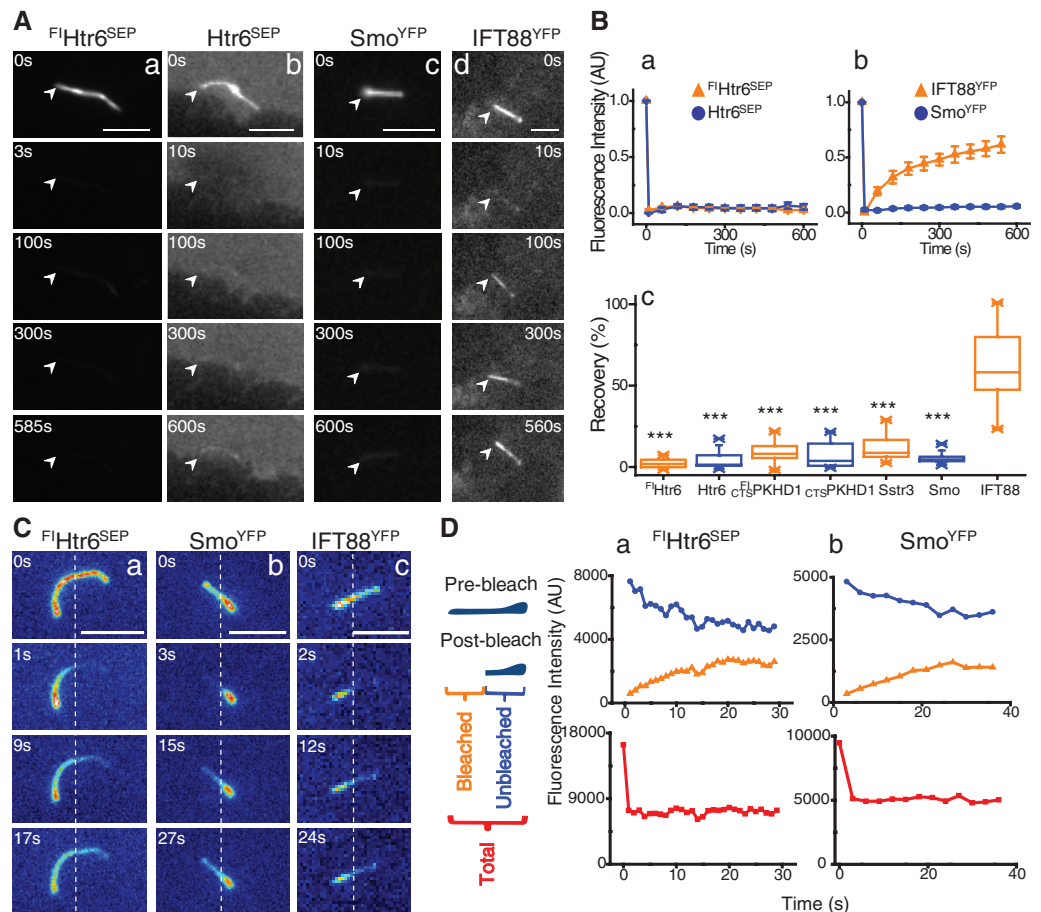


Fig. 2. (A) SEPT2 localization at the cilium of IMCD3 cells stably expressing $F^{\text{Htr6}}\text{SEPT2}$ (a), MEFs stably expressing Smo^{YFP} treated with 100 nM SAG for 24 hours (b), and IMCD3 cells stained for acetylated α -tubulin (AcTub) (c); higher magnification of boxed region shown at the bottom), and imaged with a super-resolution Optical Microscope Experimental (OMX) system using three-dimensional structured illumination (d). Scale bars, 2 μm . **(B)** SEPT2 and AcTub localization at the cilium of IMCD3 cells transiently expressing $\text{CEP164}^{\text{LAP}}$ (a), Odf2^{LAP} (b) or $\text{PeriCT}^{\text{RFP}}$ (c). Scale bars, 2 μm . (Lower panels) Fluorescence intensity profiles of protein staining from the basal body to the tip of the cilium. **(C)** IMCD3 cells grown for 24 hours without (–) or with (+) serum and stained for SEPT2, α -tubulin (α -tub), and γ -tubulin (γ -tub). Scale bars, 2 μm . **(D)** Percentage of cells with SEPT2 localized at the base of primary cilia (–serum) or pericentriolar region (+serum). Error bars represent SD of three independent experiments ($n = 60$ to 178 each; $***P < 0.0001$).

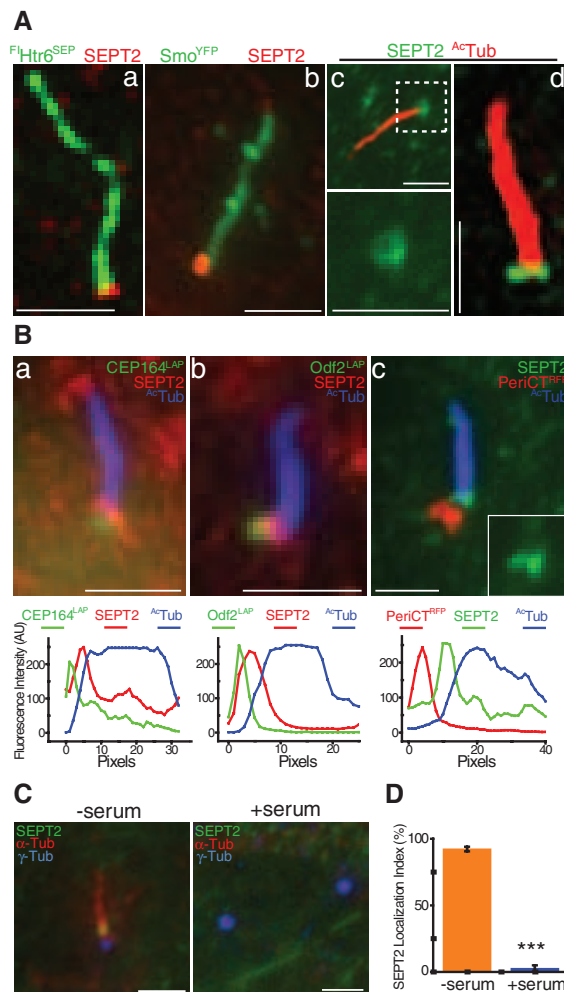
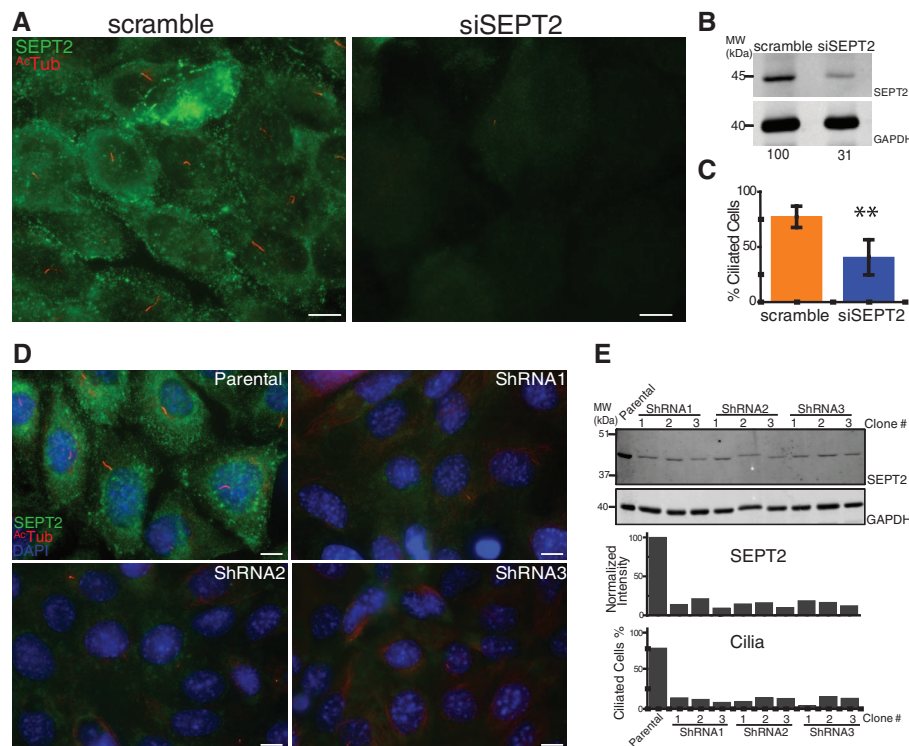


Fig. 3. (A) IMCD3 cells transfected with scrambled (scramble) or SEPT2 siRNA (siSEPT2), serum-starved to induce ciliogenesis, and stained for SEPT2 and AcTub . Scale bars, 10 μm . **(B)** Whole-cell lysates of scramble and siSEPT2 cells immunoblotted for SEPT2 and glyceraldehyde phosphate dehydrogenase (GAPDH); lower panel, SEPT2 band intensities normalized to GAPDH. **(C)** Percentage of scramble and siSEPT2 cells with a cilium $>1 \mu\text{m}$ long ($\pm$ SD of three independent experiments; $n = 101$ to 205 each; $**P = 0.0098$). **(D)** IMCD3 cells (Parental), and IMCD3 cells stably depleted of SEPT2 using shRNA1, shRNA2, or shRNA3, stained for SEPT2, AcTub , and 4',6'-diamidino-2-phenylindole (DAPI). Scale bars, 10 μm . **(E)** Whole-cell lysates of parental IMCD3 cells and three arbitrarily chosen clones of shRNA1, 2, or 3 cells immunoblotted for SEPT2 and GAPDH; lower panel, SEPT2 band intensities normalized to GAPDH, and percentage of parental and shRNA1, 2, and 3 IMCD3 cells with a cilium ($n = 115$ to 282).

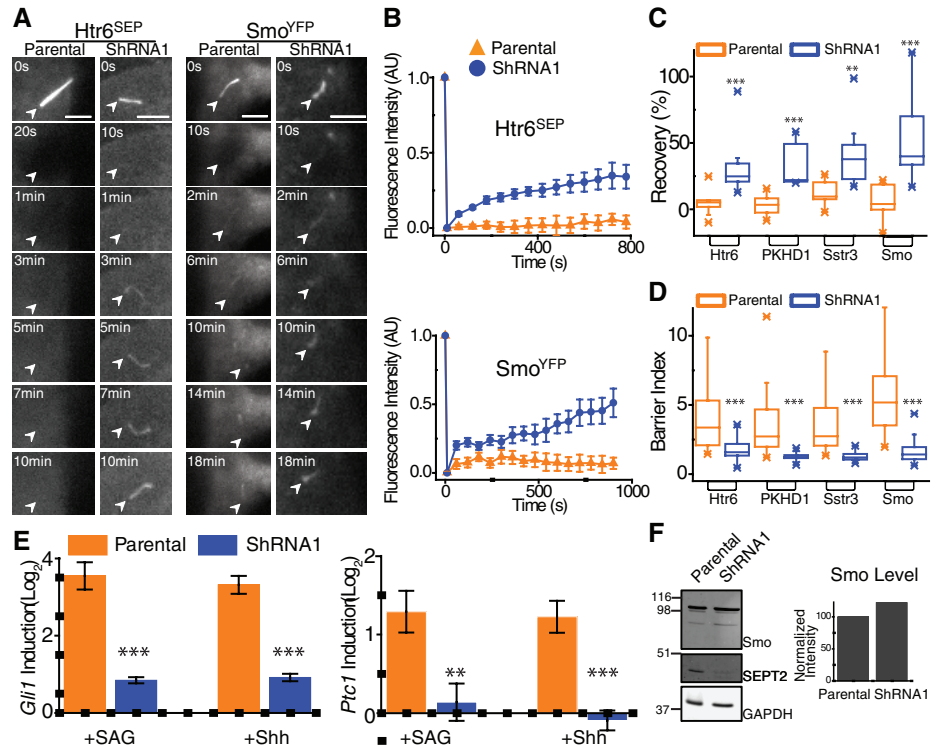


cells, and the Shh signaling pathway transducer smoothened (Smo^{YFP}) (14) in mouse embryonic fibroblasts (MEF). All of these proteins localized to the cilium, but none recovered fluorescence when the whole cilium was photobleached (Fig. 1, A and B; fig. S1, A and B; and movies S1 to S6) (15), even though their mobility was high in the periciliary membrane, as shown for Htr6^{SEP} (fig. S1C, table S1, and movie S7). In contrast, fluorescence recovery of the intraflagellar transport complex protein IFT88 ($\text{IFT88}^{\text{YFP}}$) (16, 17) was high (Fig. 1, A and B, table S1, and movie S8).

The low diffusional mobility of these ciliary membrane proteins might be due to their immobilization within the primary cilium or to the presence of a diffusion barrier at the base of the ciliary membrane. To distinguish between these possibilities, we photobleached part of the cilium and measured the fluorescence recovery of ciliary membrane proteins (Fig. 1, C and D; fig. S1, D and E; and movies S9 to S13). Strikingly, all proteins were highly mobile within the ciliary membrane (fig. S1F and table S2), although fluorescence recovery was still confined within the cilium (Fig. 1, C and D, and fig. S1, D and E). Note that constituent plasma membrane proteins (GPI^{YFP} and $\text{VSV-G}^{\text{GFP}}$) were also excluded from the ciliary membrane (fig. S2). Therefore, the diffusion of membrane proteins between the ciliary and periciliary membrane, unlike the movement of IFT88 and cytoplasmic GFP (18), is impeded at the base of the cilium by a diffusion barrier.

To identify molecular components of this ciliary membrane diffusion barrier, we focused on septins. Septins are oligomeric guanosine

Fig. 4. (A and B) FRAP of the whole cilium of parental IMCD3 (Parental) and shRNA1 IMCD3 cells transiently expressing Htr6^{SEP} or Smo^{YFP} and treated with 100 nM SAG for 24 hours. Scale bars, 5 μ m. **(B)** Kinetics of average ($\pm$ SEM) fluorescence recovery of photobleached proteins ($n = 9$ to 12). **(C)** Summary of percentage of fluorescence recovery of photobleached ciliary membrane proteins in parental and shRNA1 cells (** $P < 0.002$; *** $P < 0.0005$). **(D)** Summary of barrier index in parental and shRNA1 cells ($n = 31$ to 59; *** $P < 0.0001$). **(E)** *Gli1* (left) and *Ptc1* (right) mRNA induction in parental and shRNA1 cells after 100 nM SAG or Shh treatment for 24 hours (average of three independent experiments $\pm$ SD; ** $P = 0.005$; *** $P < 0.0007$). **(F)** Whole-cell lysates of parental and shRNA1 cells immunoblotted for Smo, SEPT2, and GAPDH; normalized Smo levels in parental and shRNA1 cells (right).



triphosphatases (GTPases) that form a diffusion barrier at the mother-bud neck of budding yeast (10, 19, 20) and localize at the annulus that may form a diffusion barrier between the middle and principal piece of the mammalian sperm flagellum (21, 22). Septin 2 (SEPT2) (23) localized to the base of the cilium at the boundary between the ciliary and periciliary membrane in IMCD3 cells (Fig. 2A and fig. S3A) and in MEFs stably expressing Smo^{YFP} (Fig. 2A). In some optical sections SEPT2 staining appeared as a ringlike structure of ~500 nm diameter (Fig. 2A), reminiscent of structures formed by recombinant septins in vitro (24), and as two fused dots (Fig. 2A and movies S14 and S15), similar to septin staining at the sperm annulus (21). In ~10% of cells, punctate SEPT2 also localized along and at the tip of the axoneme (fig. S3B).

SEPT2 localization at the base of the axoneme is similar to basal body distal appendage proteins. SEPT2, however, clearly localized between the ciliary axoneme [marked by acetylated α -tubulin (α -Tub)] and structures marked by the distal appendage protein CEP164 (25), the subdistal/distal appendage protein outer dense fiber 2 (Odf2) (26), and the pericentriolar protein pericentrin (PeriCT) (27) (Fig. 2B and fig. S3, C to E). SEPT2, but not the ciliary axoneme or these basal body proteins, was solubilized in buffer containing 0.5% TritonX-100 (fig. S3F), indicating that SEPT2, like other septins (28, 29), is associated with membrane. In addition, SEPT2 did not localize to the centrosome in nonciliated cells (Fig. 2, C and D), further indicating that it is not a constituent centrosomal protein.

To investigate whether SEPT2 is required for cilium formation and ciliary membrane barrier function, we used transient transfection with small interfering RNA (siRNA) oligonucleotides to deplete ~69% SEPT2 from serum-starved IMCD3 cells (Fig. 3, A and B). In general, cells that were completely depleted of SEPT2 lacked a cilium (Fig. 3C and fig. S4, A and B), whereas cells that were partially depleted (~54.3% depletion at the base of primary cilia) had a cilium that was significantly shorter ($3.46 \pm 0.20 \mu$ m) than controls ($4.78 \pm 0.31 \mu$ m). We also established stable IMCD3 cell lines depleted of SEPT2 using three different short hairpin RNAs (shRNAs), which reduced SEPT2 levels by 80 to 90% (Fig. 3, D and E) and resulted in a more complete defect in ciliogenesis across the cell population (Fig. 3E and fig. S4, C and D).

To investigate whether SEPT2 is part of a diffusion barrier at the base of the ciliary membrane, we measured ciliary membrane protein mobility in the 10 to 15% of the stable SEPT2-depleted IMCD3 cells (shRNA1) that had a short cilium but did not have significant SEPT2 staining near the basal body (fig. S4C). We detected a significant increase in the diffusional mobility of four ciliary membrane proteins when the whole cilium was photobleached in SEPT2-depleted cells, compared with controls (Fig. 4, A to C; fig. S5, A and B; table S3; and movies S16 to S23). Furthermore, the barrier index, defined as the ratio of mean fluorescence intensity of these proteins in the ciliary membrane to surrounding periciliary membrane, was reduced significantly in SEPT2-depleted cells compared with controls (Fig. 4D and fig. S5C). Thus, SEPT2 depletion removed

the ciliary membrane diffusion barrier in cells that could still assemble a cilium. Because the cilium was shorter in these cells, and absent in most SEPT2-depleted cells, it is possible that loss of the diffusion barrier contributed to the overall defect in ciliogenesis.

We investigated whether loss of the ciliary membrane diffusion barrier after SEPT2 depletion affected cilium-dependent receptor signal transduction. Shh signaling requires enrichment of Smo in the ciliary membrane, and signal transduction results in increased *Gli1* and *Patched1* (*Ptc1*) mRNA levels (14, 30). In SEPT2-depleted cells, Smo accumulation in cilia was reduced, although total cellular levels were unaffected (Fig. 4, D and F, and fig. S5C). After induction with Shh or Smo agonist (SAG), *Gli1* and *Ptc1* mRNA levels were reduced significantly in SEPT2-depleted cells compared with controls (Fig. 4E). Thus, the SEPT2 diffusion barrier is required for cilium-dependent Shh signal transduction.

Here, we have identified a septin-containing diffusion barrier at the base of the ciliary membrane that is required to retain receptor-signaling pathways in the primary cilium. This diffusion barrier restricts the diffusion of ciliary membrane proteins between the ciliary and periciliary membrane but permits the diffusion of ciliary transport proteins (IFT88). This implies that newly synthesized ciliary membrane proteins are most likely inserted by IFT and Bardet-Biedl Syndrome proteins into the ciliary membrane above the diffusion barrier (7). Thus, septins appear to have evolutionarily conserved roles from fungi to animals in the functional compartmentalization of membrane domains.

References and Notes

1. J. T. Eggenschwiler, K. V. Anderson, *Annu. Rev. Cell Dev. Biol.* **23**, 345 (2007).
2. W. F. Marshall, S. Nonaka, *Curr. Biol.* **16**, R604 (2006).
3. M. A. Lancaster, J. G. Gleeson, *Curr. Opin. Genet. Dev.* **19**, 220 (2009).
4. B. T. Emmer, D. Maric, D. M. Engman, *J. Cell Sci.* **123**, 529 (2010).
5. R. D. Sloboda, J. L. Rosenbaum, *J. Cell Biol.* **179**, 575 (2007).
6. G. R. Hunnicutt, M. G. Kosfischer, W. J. Snell, *J. Cell Biol.* **111**, 1605 (1990).
7. M. V. Nachury, E. S. Seeley, H. Jin, *Annu. Rev. Cell Dev. Biol.* **26** (2010), 10.1146/annurev.cellbio.042308.113337.
8. A. Musgrave *et al.*, *Planta* **167**, 544 (1986).
9. O. V. Vieira *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 18556 (2006).
10. F. Caudron, Y. Barral, *Dev. Cell* **16**, 493 (2009).
11. Q. Hu, W. J. Nelson, E. T. Spiliotis, *J. Biol. Chem.* **283**, 29563 (2008).
12. N. F. Berbari, A. D. Johnson, J. S. Lewis, C. C. Askwith, K. Myktyyn, *Mol. Biol. Cell* **19**, 1540 (2008).
13. J. A. Folliot, L. Li, Y. Vucica, G. J. Pazour, *J. Cell Biol.* **188**, 21 (2010).
14. R. Rohatgi, L. Milenkovic, M. P. Scott, *Science* **317**, 372 (2007).
15. K. Huang *et al.*, *J. Cell Biol.* **179**, 501 (2007).
16. P. V. Tran *et al.*, *Nat. Genet.* **40**, 403 (2008).
17. G. J. Pazour *et al.*, *J. Cell Biol.* **151**, 709 (2000).
18. P. D. Calvert, W. E. Schiesser, E. N. Pugh Jr., *J. Gen. Physiol.* **135**, 173 (2010).
19. Y. Barral, V. Mermall, M. S. Mooseker, M. Snyder, *Mol. Cell* **5**, 841 (2000).
20. P. A. Takizawa, J. L. DeRisi, J. E. Wilhelm, R. D. Vale, *Science* **290**, 341 (2000).
21. M. Ihara *et al.*, *Dev. Cell* **8**, 343 (2005).
22. H. Kissel *et al.*, *Dev. Cell* **8**, 353 (2005).
23. E. T. Spiliotis, S. J. Hunt, Q. Hu, M. Kinoshita, W. J. Nelson, *J. Cell Biol.* **180**, 295 (2008).
24. M. Kinoshita, C. M. Field, M. L. Coughlin, A. F. Straight, T. J. Mitchison, *Dev. Cell* **3**, 791 (2002).
25. S. Graser *et al.*, *J. Cell Biol.* **179**, 321 (2007).
26. H. Ishikawa, A. Kubo, S. Tsukita, S. Tsukita, *Nat. Cell Biol.* **7**, 517 (2005).
27. A. K. Gillingham, S. Munro, *EMBO Rep.* **1**, 524 (2000).
28. J. Zhang *et al.*, *Curr. Biol.* **9**, 1458 (1999).
29. Y. Tanaka-Takiguchi, M. Kinoshita, K. Takiguchi, *Curr. Biol.* **19**, 140 (2009).
30. K. C. Corbit *et al.*, *Nature* **437**, 1018 (2005).
31. We thank M. Kinoshita, S. Munro, G. J. Pazour, K. Myktyyn, D. R. Beier, C. Janke, J. Christianson, R. Kopito, and T. Stearns for reagents, and J. Atkins (Applied Precision, Inc.) for technical support. Supported by NIH grant GM35527 (W.J.N.), Department of Defense Breast Cancer Research Program Predoctoral Fellowship BC083077 (Q.H.), and Drexel CURE award from the State of Pennsylvania Tobacco Settlement Funds (E.T.S.). Supported in part by grants (M.V.N.) from the American Heart Association, the March of Dimes, NIH (GM089933), and the Sloan, Klingenstein and Searle Foundations. L.M. is a Research Specialist, and M.P.S. an Investigator of the Howard Hughes Medical Institute.

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1191054/DC1
Materials and Methods

Figs. S1 to S5

Tables S1 to S3

References

Movies S1 to S23

19 April 2010; accepted 8 June 2010

Published online 17 June 2010;

10.1126/science.1191054

Include this information when citing this paper.

Integrative Modeling Defines the Nova Splicing-Regulatory Network and Its Combinatorial Controls

Chaolin Zhang,* Maria A. Frias, Aldo Mele, Matteo Ruggiu, Taesun Eom, Christina B. Marney, Huidong Wang, Donny D. Licatalosi, John J. Fak, Robert B. Darnell*

The control of RNA alternative splicing is critical for generating biological diversity. Despite emerging genome-wide technologies to study RNA complexity, reliable and comprehensive RNA-regulatory networks have not been defined. Here, we used Bayesian networks to probabilistically model diverse data sets and predict the target networks of specific regulators. We applied this strategy to identify ~700 alternative splicing events directly regulated by the neuron-specific factor Nova in the mouse brain, integrating RNA-binding data, splicing microarray data, Nova-binding motifs, and evolutionary signatures. The resulting integrative network revealed combinatorial regulation by Nova and the neuronal splicing factor Fox, interplay between phosphorylation and splicing, and potential links to neurologic disease. Thus, we have developed a general approach to understanding mammalian RNA regulation at the systems level.

RNA-binding proteins (RBPs) regulate alternative splicing (AS) and processing of RNA to generate biological complexity (1). Inferring RNA target networks regulated by these splicing factors may provide general insights into the mechanisms of regulation and their role in disease (2–5). Several global approaches have recently been applied toward this aim (2), including bioinformatic predictions driven by analysis of RBP motifs (6–8), profiling of RNA isoforms based on splicing microarrays

(9–11) or RNA-Seq (12–14), and biochemical footprints derived from high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) (9, 15). These methods have been applied to identify and genetically validate ~90 alternative exons regulated by Nova1/2 (9, 10), a family of neuron-specific splicing factors. Nova regulates a biologically coherent set of transcripts encoding synaptic proteins (10), and an RNA-regulatory map predicts that Nova-regulated splicing is position dependent, such that alternative exons are included when Nova binds to downstream introns and are excluded via binding within the exons or to upstream introns (9, 16).

Each of these methods is limited in its signal-to-noise ratio and scope: RBP motifs generally

have very low sequence specificity [e.g., YCAY for Nova, ~1 site per 64 nucleotides (nt)]; microarray or RNA-Seq data are noisy at the exon level beyond a small set of top candidates and are correlative in nature; and biochemical protein-RNA interactions do not necessarily imply functional regulation. Consequently, only a small set of targets have been confidently identified for most splicing factors (4, 17). An alternative strategy is to integrate multiple sources of information, so that individually weak bits of evidence can be combined to generate confident predictions, as demonstrated in studies of protein-protein interactions (18) and transcription factor networks (19). Here, we set out to develop such an integrative approach to probabilistically model a diverse set of genomic, experimental, and evolutionary data, using Bayesian networks to define and understand the function of RNA networks.

We studied the Nova splicing-regulatory network as an exemplar and compiled four types of data important for inferring direct Nova-RNA interactions coupled with defined Nova-dependent AS events: (i) 279,631 CLIP tag clusters, ranked by peak height, derived from 20 independent HITS-CLIP experiments (figs. S1 and S2, table S1, and datasets S1 and S2); (ii) 841,501 Nova-binding sites (YCAY clusters) bioinformatically predicted and scored from the clustering, accessibility, and conservation of YCAY elements (fig. S3); (iii) four splicing-microarray data sets comparing wild-type and Nova knockout (KO) brains, which detected 1331 exons showing significant Nova-dependent splicing, in addition to many exons with moderate but potentially functional changes (fig. S4 and table S2); and (iv) evolutionary signatures of regulated splicing, including conservation of AS in humans or rats, and preservation of reading frame (20). Each individual data set suggested

Laboratory of Molecular Neuro-Oncology, Howard Hughes Medical Institute, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA.

*To whom correspondence should be addressed. E-mail: czhang@rockefeller.edu (C.Z.); darnell@rockefeller.edu (R.B.D.)

a large number of informative but noisy candidates, arguing for the importance of rigorous data integration.

To probabilistically weigh and combine these data sets, we designed a Bayesian network for each of seven types of AS events: cassette exons (an exon is included or skipped), tandem cas-

sette exons, mutually exclusive exons (one of two exons is included), alternative 5' and 3' splice sites, and alternative poly(A) usage coupled with 5' or 3' splice site choices (table S5); each AS event represents an observation of the Bayesian network. Using cassette exons as an example, the network included 17 nodes (variables) con-

ected by edges reflecting the causal relationships between variables (Fig. 1A and table S3). The strength of YCA clusters determines Nova binding (a binary hidden variable) in upstream introns, exons, or downstream introns, which in turn determines the probability of observing binding footprints by HITS-CLIP. The combi-

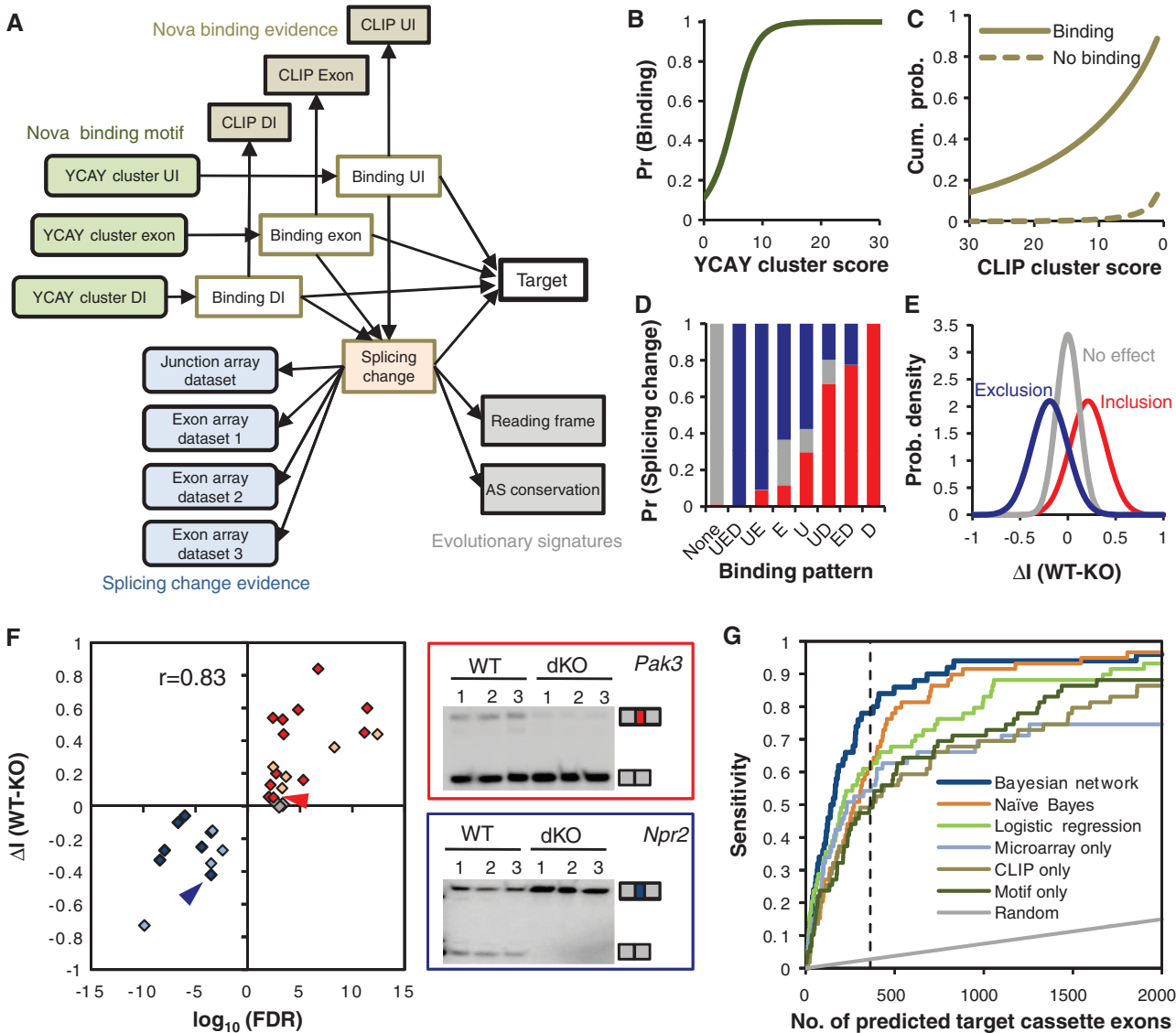


Fig. 1. Integrative prediction of Nova targets using a Bayesian network. The Bayesian network (BN) for cassette exons is shown. (A) Design of the BN. The 17 nodes (variables) model four types of data, including YCAY clusters and CLIP clusters in each cassette exon or flanking upstream (UI) and downstream introns (DI), splicing microarrays comparing wild-type (WT) and Nova KO brains, and evolutionary signatures. See table S3 for more details. (B to E) Estimated conditional probability distributions derived from the BN. (B) The probability of Nova binding to regions with varying YCAY cluster scores across all regions. (C) The cumulative probability of CLIP cluster scores across all regions with or without inferred Nova binding. (D) The probability of exons showing Nova-dependent inclusion (red), exclusion (blue), or no effect in comparisons of WT versus Nova KO brain transcripts, given the indicated combinatorial Nova binding patterns in exon (E), upstream (U) and downstream (D) introns. (E) The distribution of proportional splicing changes between WT and Nova KO brain RNA [ΔI (10)] measured by exon-junction arrays for exons with inferred Nova-dependent inclusion, exclusion, or without Nova regula-

tion. (F) A summary of reverse transcriptase–polymerase chain reaction (RT-PCR) analysis for the 31 exons tested in WT versus NOVA2KO or Nova1/2 double KO (dKO) brains. Twenty-two exons have Nova-regulated inclusion or exclusion ($P < 0.05$; t test), and those without significant changes tested in this study are shown in red, blue, and grey, respectively; 9 previously validated exons are similarly shown in light red and light blue, respectively. The correlation between prediction confidence and the magnitude of splicing change is indicated. For two representative BN-predicted exons (arrowheads in the scatter plot), gel images are shown with the two isoforms including or excluding the regulated exon indicated (right panel). (G) Comparison of Nova target prediction by different methods (BN, naïve Bayes, and logistic regression) or using individual data sets (microarrays comparing embryonic day 18.5 WT versus Nova1/2 dKO brains, CLIP clusters, and YCA clusters). Each curve represents the prediction sensitivity with varying stringency; the performance of random predictions is also shown. The dotted line indicates the top 363 predictions.

natorial action of Nova binding in one or more regions dictates the splicing outcome (another hidden variable), as reflected in microarray measurements and evolutionary signatures. With this predetermined network structure, the parameters of the model (conditional probability distributions) were learned from a subset of training cassette exons, including 50 previously validated targets (20).

Unlike “black box” predictions, the learned model parameters provide interpretable and novel insights into Nova splicing regulation (Fig. 1, B to E, and fig. S5). For example, the model confirmed and extended the previously defined RNA-regulatory map (9, 16), quantifying it and predicting the combinatorial action of Nova binding in multiple regions: Nova binding in exons or upstream introns alone results in exon exclusion with a probability of ~0.6,

which increases to >0.9 if Nova binds to both regions (Fig. 1D).

We prospectively applied the model to 13,357 annotated cassette exons (20). Each exon was assigned three probabilities that measure Nova-regulated exon inclusion, exclusion, and absence of direct regulation, respectively, from which a false-discovery rate (FDR) was estimated. After ensuring that the model was not overfit by 10-fold cross-validation (fig. S6), we predicted 363 cassette exons as direct Nova targets, with a stringent FDR of ≤ 0.01 , and more broadly, 588 Nova-regulated AS events (table S5) when applied to all types of AS events (fig. S7).

We also searched novel exons with high sequence conservation and exons whose AS pattern was missed in our database (fig. S8 and table S4) (20). This search conservatively identified 76 additional exons as Nova targets.

Hence, the final Nova target network included 698 AS events from 358 genes, among which 610 events (87%) represent novel predictions (table S5).

To evaluate the quality of the network, we performed unbiased experimental validation. Intersecting the Bayesian network-predicted exons with a collection of well-studied alternative exons (AEDB) (21) yielded a manageable set of 31 non-redundant exons, whose confidence scores are distributed very uniformly (median rank: 288 of 588; Fig. 1F and table S6). Among these, nine are previously validated Nova targets, and 19 of the remaining 22 exons were validated by comparing AS in wild-type and Nova KO brains ($P < 0.05$, t test, $n = 6$) (Fig. 1F and fig. S9). In addition, we validated 8 of 9 novel exons tested (fig. S10), yielding an overall validation rate of ~90% (28 of 31 or 36 of 40). Combined with its

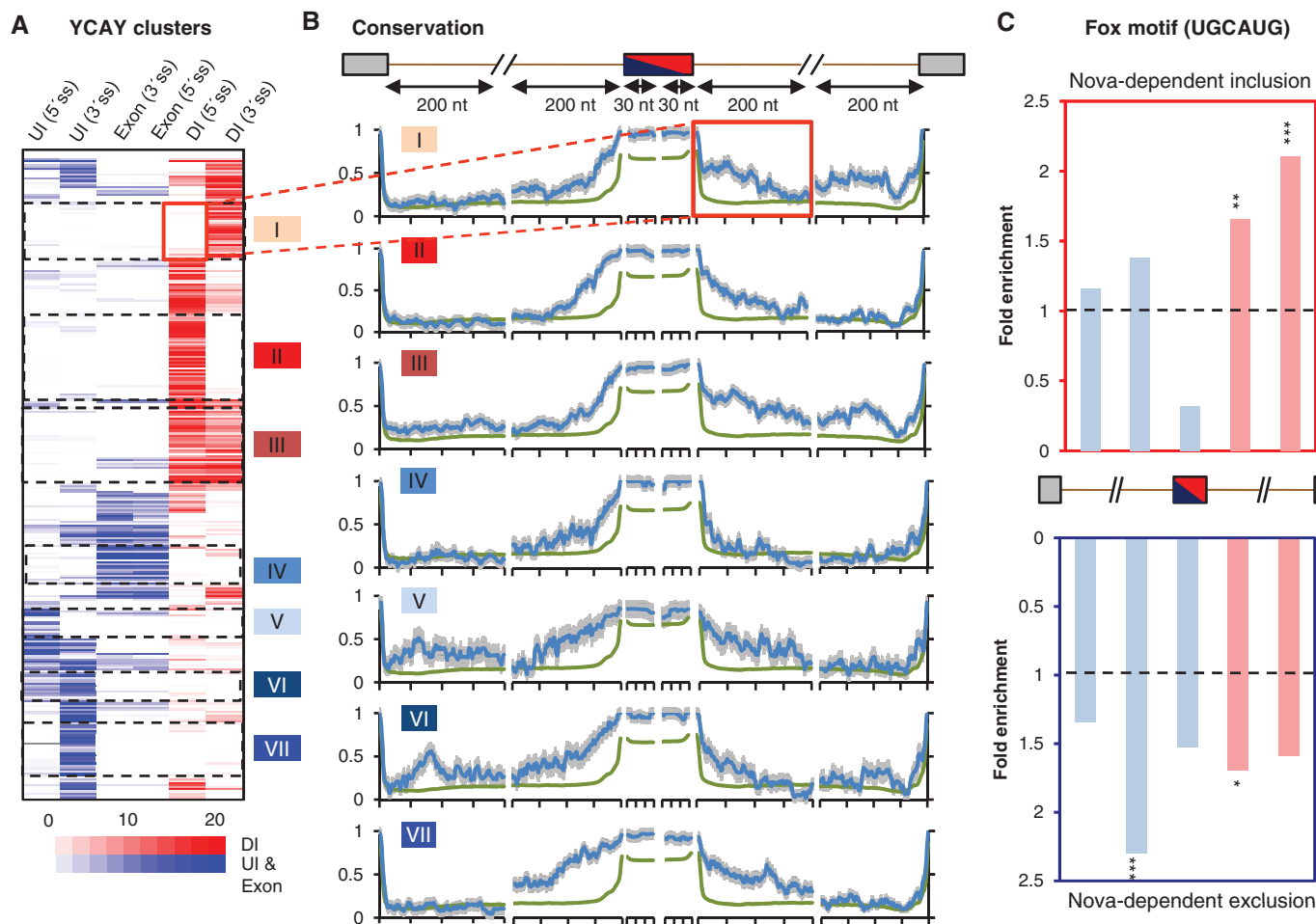


Fig. 2. Combinatorial regulation of Nova target exons. **(A)** Hierarchical clustering of 325 nonredundant Nova-regulated cassette exons using six regional YCAY cluster scores in exon, UI, and DI relative to alternative splice sites. The position of DI and UI or exonic YCAY clusters is predicted to dictate Nova-regulated alternative exon inclusion (red) or exclusion (blue). Seven clusters of exons with distinct Nova-binding patterns are shown. **(B)** Sequence conservation scores across 20 mammalian species were extracted for 30-nt exonic regions near 5' or 3' splice site of the regulated exon, or for 200-nt intronic regions near all four possible splice sites, as indicated in the

cartoon. The average conservation profile is shown for each cluster in **(A)** (blue), using all cassette exons as a control (green). Error bars represent standard errors. The flanking intronic region downstream of the cassette exon in cluster I is highlighted. **(C)** The enrichment of the Fox motif (UGCAUG) in exons with Nova-regulated inclusion (top) or exclusion (bottom), as compared to control cassette exons. Fox-binding sites predicted to dictate Fox-regulated exon inclusion (DI) or exclusion (UI or exon) are represented by red and blue bars, respectively. Statistical significance is derived from a χ^2 test ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

high sensitivity in predicting 58 of 77 (75%) previously validated targets (39/50 = 78% for cassette exons), the accuracy of our network compares favorably with previous studies, which obtained substantially lower validation rates or more limited sets of candidates (9, 10, 22, 23).

The Bayesian network analysis successfully integrated information from multiple types of data, predicting a substantial portion of targets missed by analysis of individual data sets or by other machine learning algorithms (20). For example, analysis of the 363 top target cassette exons predicted from microarrays, CLIP clusters, or YCA clusters alone achieved 49 to 54% sensitivity and an estimated validation rate of 54 to 61%, compared to 75 to 78% sensitivity and ~90% validation of the Bayesian network (Fig. 1G and fig. S11). Integration of microarray data, CLIP clusters, and YCA clusters by naïve Bayes or logistic regression produced moderate improvement, with 61% sensitivity and an estimated validation rate of 65 to 67% (Fig. 1G and fig. S11, C and E). These observations underscore the effectiveness of our integrative strategy.

The comprehensive list of Nova targets makes it possible to correlate the positions of Nova-binding sites with sequence conservation pro-

files (Fig. 2, A and B, and dataset S3). Although Nova-binding sites are generally conserved (2), unexpectedly, we identified additional conserved regions in regulated exons outside Nova-binding sites (Fig. 2, A and B), suggesting the presence of additional regulatory elements. To search for specific RBPs that might dictate coordinated combinatorial regulation with Nova, we examined putative splicing-regulatory elements derived from brain-specific AS exons (12). The well-characterized Fox-binding element (UGCAUG) (24) was enriched in both ends of downstream introns (1.7-fold, $P = 0.002$ for 5' end; 2.1-fold, $P = 4.7 \times 10^{-6}$ for 3' end; χ^2 test) that border cassette exons showing Nova-dependent inclusion, and upstream introns near 3' splice sites (2.3 fold, $P = 1.8 \times 10^{-5}$; χ^2 test) of cassette exons showing Nova-dependent exclusion (Fig. 2C and fig. S12). Furthermore, 106 of the 698 Nova target AS events were candidate Fox targets in the brain, with highly conserved UGCAUG elements (7), indicating that ~15% of Nova targets may be under Nova and Fox combinatorial control (5.5-fold enrichment, $P < 10^{-46}$, Fisher's exact test). Because Fox-regulated splicing is defined by a position-dependent RNA-regulatory map similar to that for Nova (7, 25), these observations suggest that additive or synergistic actions of

Nova and Fox may be favored over antagonistic actions.

To experimentally address the bioinformatic prediction of Nova and Fox combinatorial regulation, we examined alternative splicing of several candidate exons (20). One of these, *Gabrg2* exon 9, is regulated by Nova through a strong YCA cluster (score = 20) ~80 nt upstream of the 3' splice site of intron 9 (Fig. 3A), as determined by mutagenesis analysis (26). An independent mutation ~30 nt downstream of exon 9 disrupted the basal level of exon 9 inclusion independent of Nova expression (26). Further examination revealed that this mutation fortuitously disrupted a very conserved Fox-binding element (Fig. 3A). To test if Nova and Fox exhibit combinatorial regulation on this exon, we transfected increasing amounts of Nova1 and Fox2, alone or in combination, into human embryonic kidney 293T cells, together with a minigene consisting of sequences between exon 8 and exon 10 (Fig. 3B). Either protein alone induced a dosage-dependent inclusion of exon 9, confirming that this exon is regulated by Nova and Fox individually. Simultaneous expression of smaller amounts of both proteins markedly increased the inclusion level from <5% to 26%, indicating a synergistic effect of Nova and Fox in splicing

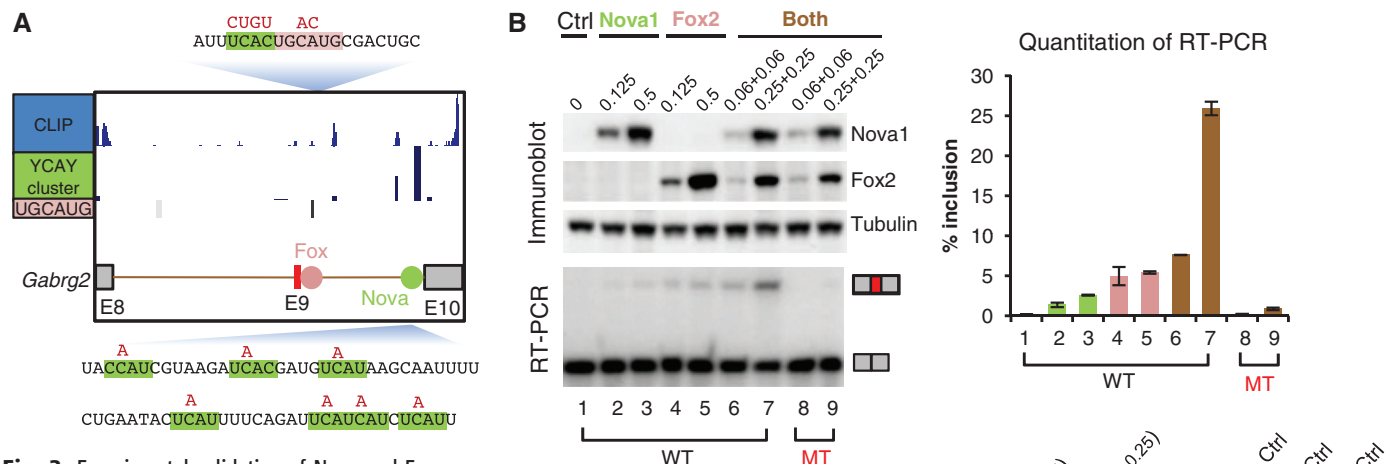


Fig. 3. Experimental validation of Nova and Fox combinatorial regulation. (A) Schematic representation of exon 8 (E8) to exon 10 (E10) of the *Gabrg2* minigene (26). The CLIP tags, YCA clusters, and UGCAUG elements are shown above the cartoon. Sequences flanking Nova (shaded in green) and Fox (shaded in red) binding sites are shown. Mutations used to disrupt the Nova and Fox binding sites are indicated above the sequence. (B) After transfection of 293T cells with the *Gabrg2* minigene in the presence of the indicated amounts (in μ g) of control (Ctrl), Nova1, or Fox2 expression plasmids, cells were analyzed for the indicated proteins by immunoblot and for *Gabrg2* E9 splicing by RT-PCR with primers flanking E9. RT-PCR yielded the larger E9 included and smaller E9 excluded isoforms, as indicated (middle panel), and the inclusion level was quantitated in the bar graph (right panel); error bars represent standard errors estimated from two biological replicates. (C) Two additional examples of exons under Nova1 and Fox2 combinatorial regulation. For each panel, the AS region, CLIP tags, YCA clusters, and UGCAUG elements are shown as in (A). The RT-PCR

analysis is shown in the middle, with alternative isoforms indicated. The four lanes represent control cells, and cells transfected with Nova1 (0.5 μ g), Fox2 (0.5 μ g), and both proteins (0.25 μ g + 0.25 μ g), respectively. The quantitated splicing changes (ΔI) are shown on the right, with averages and standard errors estimated from four replicates.

regulation. This synergistic regulation is direct, because mutations of their binding sites reduced exon 9 inclusion to basal levels even in the presence of both proteins. These observations suggest a model in which the binding of Fox and Nova in cis is able to synergize, perhaps by inducing a looping of the intron and thus the tethering of exons 9 and 10.

Altogether, we validated seven exons showing splicing regulation by both proteins, through synergistic (*Gabrg2* and *Mtap7d2*), additive (*Numb*, *Syne2*, and *Pbrm1*), or antagonistic (*Arhgef12* and *Alcam*) actions (Fig. 3, B and C, and fig. S13). In all seven cases, the splicing outcomes can be predicted from a combinatorial RNA-regulatory map derived by superposing the map for each individual protein (fig. S13), offering a means of understanding the spatial and temporal control of RNA complexity.

Nova regulates AS of transcripts encoding synaptic proteins that themselves interact with each other (10). The comprehensive network confirmed and extended this observation, using GO (Gene Ontology) term and KEGG (Kyoto Encyclopedia of Genes and Genomes) path-

way analysis (tables S7 and S8). Nonetheless, it has been unclear exactly how Nova-regulated AS might affect such interactions. Analysis of protein annotations revealed that about half of Nova target transcripts encoded phosphoproteins, a 1.7-fold enrichment compared to brain-expressing AS genes ($P < 10^{-13}$, Fisher's exact test) (Fig. 4A) (20). Furthermore, Nova-regulated exons within these transcripts themselves encoded experimentally determined phosphorylation sites much more frequently compared with constitutive or overall alternative exons (>2.4 -fold, $P < 10^{-12}$; Fisher's exact test; Fig. 4B), or more strictly with nontarget brain-specific AS exons (1.7-fold, $P < 0.0004$; Fisher's exact test; Fig. 4B) (12, 20). Similar observations were obtained with a more stringent subset of phosphorylation sites experimentally determined in the brain and thus most relevant for Nova function (20) (fig. S14). Moreover, Nova target genes included 25 kinases and 9 phosphatases, a 2.5-fold enrichment compared to all brain-expressing genes ($P = 10^{-5}$, Fisher's exact test). Thus, Nova directly affects the in vivo phosphorylation patterns of brain proteins via AS regulation, a two-layered control to modulate downstream protein-protein interactions and physiological functions (Fig. 4C and table S6).

Finally, the comprehensiveness of the network suggests new relationships to physiology and disease. A subset of newly predicted Nova-regulated exons are known to be functionally significant, and in some cases are essential for viability [e.g., the switch of *Snap25* exon 5a/5b (27); table S6]. Of the 358 Nova target genes, 88 are currently implicated in genetic diseases (1.5-fold enrichment compared to brain-expressing genes, $P < 5 \times 10^{-4}$, Fisher's exact test; table S9) (20), including neurologic disorders such as mental retardation, epilepsy, and autism. *Fox1* (*A2BP1*) itself is an autism susceptibility gene (28). Moreover, 8.5% genes predicted to be regulated by both Nova and Fox (on the same or different exons) are implicated in autism, compared to 3.3 to 3.4% for genes targeted by Nova or Fox alone ($P < 0.02$, χ^2 test) and 1.2% in all brain-expressing genes ($P = 10^{-7}$, Fisher's exact test) (20). Thus, coordinated RNA regulation may be susceptible to disruptions in complex multigenic neurologic diseases. Although placing discrete exons and genes in the Nova (and Fox) target networks already points ways toward greater understanding of RNA regulation and disease mechanisms, the functions encoded by most AS exons remain to be characterized.

Recent advances in machine learning with sequence motifs and other RNA features are beginning to derive general rules relevant to tissue-specific splicing regulation (8). These efforts are complemented and extended by the network analysis presented here, which sums multiple types of data to generate highly accurate and global predictions of specific RBP-target regulatory interactions. This strategy should

improve splicing code fidelity and provide a guide to prioritize further functional studies. Taken together, the integrative network analysis has the potential to fill gaps between the delineation of alternative RNA processing, its underlying regulatory mechanisms, and its biological significance.

References and Notes

1. T. W. Nilsen, B. R. Graveley, *Nature* **463**, 457 (2010).
2. D. D. Licatalosi, R. B. Darnell, *Nat. Rev. Genet.* **11**, 75 (2010).
3. T. A. Cooper, L. Wan, G. Dreyfuss, *Cell* **136**, 777 (2009).
4. Q. Li, J.-A. Lee, D. L. Black, *Nat. Rev. Neurosci.* **8**, 819 (2007).
5. D. D. Licatalosi, R. B. Darnell, *Neuron* **52**, 93 (2006).
6. A. Galarneau, S. Richard, *Nat. Struct. Mol. Biol.* **12**, 691 (2005).
7. C. Zhang et al., *Genes Dev.* **22**, 2550 (2008).
8. Y. Barash et al., *Nature* **465**, 53 (2010).
9. D. D. Licatalosi et al., *Nature* **456**, 464 (2008).
10. J. Ule et al., *Nat. Genet.* **37**, 844 (2005).
11. J. C. Castle et al., *Nat. Genet.* **40**, 1416 (2008).
12. E. T. Wang et al., *Nature* **456**, 470 (2008).
13. Q. Pan, O. Shai, L. J. Lee, B. J. Frey, B. J. Blencowe, *Nat. Genet.* **40**, 1413 (2008).
14. B. J. Blencowe, S. Ahmad, L. J. Lee, *Genes Dev.* **23**, 1379 (2009).
15. R. B. Darnell, HITS-CLIP: Panoramic views of protein-RNA regulation in living cells. *Wiley Interdiscipl. Rev. RNA* 10.1002/wrna.31 (2010).
16. J. Ule et al., *Nature* **444**, 580 (2006).
17. M. Chen, J. L. Manley, *Nat. Rev. Mol. Cell Biol.* **10**, 741 (2009).
18. R. Jansen et al., *Science* **302**, 449 (2003).
19. Y. Wang, X.-S. Zhang, Y. Xia, *Nucleic Acids Res.* **37**, 5943 (2009).
20. Materials and methods are available as supporting material on Science Online.
21. S. Stamm et al., *Nucleic Acids Res.* **34**, D46 (2006).
22. P. L. Boutz et al., *Genes Dev.* **21**, 1636 (2007).
23. Y. Xue et al., *Mol. Cell* **36**, 996 (2009).
24. J. L. Ponthier et al., *J. Biol. Chem.* **281**, 12468 (2006).
25. G. W. Yeo et al., *Nat. Struct. Mol. Biol.* **16**, 130 (2009).
26. B. K. Dredge, R. B. Darnell, *Mol. Cell Biol.* **23**, 4687 (2003).
27. C. Bark et al., *J. Neurosci.* **24**, 8796 (2004).
28. C. L. Martin et al., *Am. J. Med. Genet. B. Neuropsychiatr. Genet.* **144B**, 869 (2007).
29. We thank A. R. Krainer for Fox1/2 expression constructs, S. Dewell for Illumina sequencing, and all Darnell lab members for helpful discussions. This work was supported by grants from the NIH to R.B.D. (NS34389) and the Rockefeller University Hospital CTSA (UL1 RR024143). R.B.D. is an Investigator of the Howard Hughes Medical Institute. The HITS-CLIP and microarray data have been deposited to the National Center for Biotechnology Information Sequence Read Archive (SRA019982) and Gene Expression Omnibus (GSE22115) databases, respectively.

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1191150/DC1
Materials and Methods
Figs. S1 to S14
Tables S1 to S9
References
Datasets S1 to S3

20 April 2010; accepted 10 June 2010

Published online 17 June 2010;

10.1126/science.1191150

Include this information when citing this paper.

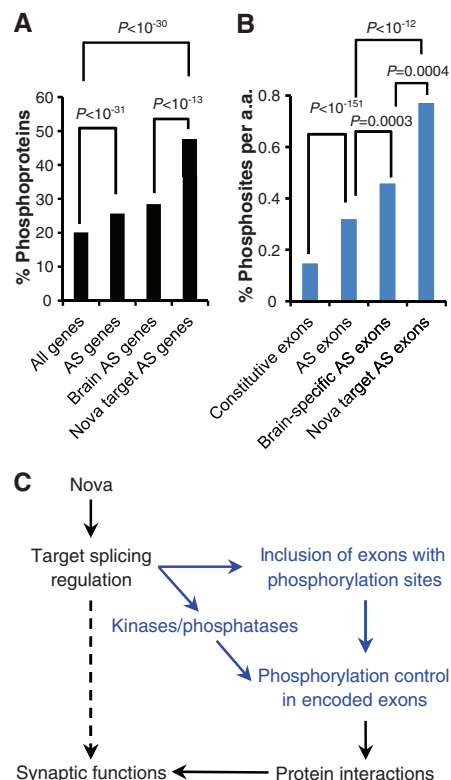


Fig. 4. Nova target AS switches protein phosphorylation. **(A)** Percentage of phosphoproteins for different sets of genes. **(B)** Percentage of experimentally determined phosphorylation sites per amino acid for different sets of exons. Different groups in **(A)** and **(B)** were compared by Fisher's exact test. **(C)** A model of Nova AS regulation to control protein phosphorylation patterns, a mechanism to modulate downstream protein-protein interactions and synaptic functions.

Dnmt3a-Dependent Nonpromoter DNA Methylation Facilitates Transcription of Neurogenic Genes

Hao Wu,^{1*} Volkan Coskun,² Jifang Tao,² Wei Xie,³ Weihong Ge,¹ Kazuaki Yoshikawa,⁴ En Li,⁵ Yi Zhang,⁶ Yi Eve Sun^{1,2*}

DNA methylation at proximal promoters facilitates lineage restriction by silencing cell type-specific genes. However, euchromatic DNA methylation frequently occurs in regions outside promoters. The functions of such nonproximal promoter DNA methylation are unclear. Here we show that the *de novo* DNA methyltransferase Dnmt3a is expressed in postnatal neural stem cells (NSCs) and is required for neurogenesis. Genome-wide analysis of postnatal NSCs indicates that Dnmt3a occupies and methylates intergenic regions and gene bodies flanking proximal promoters of a large cohort of transcriptionally permissive genes, many of which encode regulators of neurogenesis. Surprisingly, Dnmt3a-dependent nonproximal promoter methylation promotes expression of these neurogenic genes by functionally antagonizing Polycomb repression. Thus, nonpromoter DNA methylation by Dnmt3a may be used for maintaining active chromatin states of genes critical for development.

De novo DNA methyltransferases Dnmt3a and Dnmt3b and maintenance methyltransferase Dnmt1 covalently modify mammalian genomes by cytosine methylation, an epigenetic mark that is essential for normal development and primarily occurs at CpG dinucleotides (1). Most CpG-rich regions (CpG islands) overlap with proximal promoters, where DNA methylation is linked to gene silencing (2). However, unbiased genome-wide analyses have shown that DNA methylation is widespread across the euchromatic portion of mammalian genomes and predominantly takes place in regions outside proximal promoters, including intergenic regions and gene bodies (2, 3), where tissue-specific DNA methylation is frequently localized (4). Deletion of Dnmt1 or treatment with Dnmt inhibitors in dividing somatic cells can lead to global DNA hypomethylation, chromosomal instability, and compromised cell-cycle progression, thereby hindering functional analysis of DNA methylation in self-renewing tissue-specific stem cells. Unlike Dnmt1 or Dnmt3b (the deletion of which, in mice, leads to early embryonic lethality), Dnmt3a is not required for maintaining methylation at heterochromatic repeat regions, and *Dnmt3a*-null mice appear to be grossly normal at birth (5, 6). However, mice lacking *Dnmt3a* do acquire develop-

mental defects postnatally and die prematurely (5), suggesting a specific role for Dnmt3a in regulating euchromatic methylation and postnatal development.

The function of DNA methylation in the postnatal brain is of particular interest, because brain-specific deletion of DNA methylation-related machineries results in postnatal neurodevelopmental abnormalities and premature death in mice (7, 8). Multiple isoforms of Dnmt3a/3b are expressed in mouse embryonic stem (ES) cells, but only the Dnmt3a full-length variant is expressed in diverse regions of the postnatal brain (7, 9), including subependymal/subventricular zones (SEZ/SVZ) of the forebrain and the hippocampal dentate gyrus (fig. S1), two neurogenic regions in which neural stem cells (NSCs) persist throughout life (10). In *Dnmt3a*-null mice, quantification of doublecortin (Dcx)-positive immature neurons demonstrated that postnatal neurogenesis at both neurogenic zones was impaired (Fig. 1, A and B, and fig. S2) (11). However, the decrease in the number of newborn neurons was unlikely to be caused by changes in proliferation and/or survival of postnatal NSCs (fig. S3, A and B). To further evaluate the function of Dnmt3a in NSCs, we derived monolayer NSC cultures from wild-type (WT) and *Dnmt3a*-null [knockout (KO)] mice. Though Dnmt3a was dispensable for maintaining NSCs in an undifferentiated and proliferating state (fig. S3, C to F), upon mitogen withdrawal, differentiating *Dnmt3a*-null cells produced more than 10-fold fewer Tuj1+/Map2+ neurons (Fig. 1, C and D) and more glial cells, as compared with WT cells (fig. S4). To exclude the possibility that impaired neurogenic potential of *Dnmt3a*-null NSCs resulted from secondary effects after long-term *Dnmt3a*-deletion, postnatal NSCs were isolated from mice homozygous for floxed alleles of *Dnmt3a* (*Dnmt3a*^{fllox/fllox}) (12) and were infected with control or Cre-expressing lentiviruses (fig. S5, A and B). Differentiation analyses indicated that acute *Dnmt3a*-deletion (5-day) also impaired

neuronal differentiation (fig. S5C). Moreover, the neurogenesis deficit in *Dnmt3a*-null cultures was rescued by re-expressing physiological levels of WT Dnmt3a, but not catalytically inactive mutant Dnmt3a (Fig. 1D and fig. S6, A and B) (13). Expression of other Dnmt proteins and global DNA methylation patterns remained largely unchanged in *Dnmt3a*-null and rescued NSCs (fig. S6, B to D). Thus, specific and cell-autonomous functions of Dnmt3a are required for neurogenesis from postnatal NSCs.

To investigate the mechanism underlying Dnmt3a-dependent neurogenesis, we mapped genome-wide Dnmt3a occupancy in postnatal NSCs by chromatin immunoprecipitation (ChIP) (N^o Ab in fig. S1B) combined with whole-genome tiling microarrays (ChIP-chip). We identified 30,417 Dnmt3a binding-sites with high-confidence (false discovery rate < 1%). Quantitative polymerase chain reaction (qPCR) analysis confirmed that all 20 randomly selected loci showed significant enrichment for Dnmt3a (fig. S7A). Control ChIP-chip experiments in *Dnmt3a*-null NSCs did not yield specific enrichment (fig. S8A), confirming the specificity of Dnmt3a ChIP results. Most Dnmt3a binding sites were located in gene-rich euchromatic regions, as 80.7% of all binding sites were within gene bodies or 50-kb intergenic regions up- and downstream of annotated genes (fig. S9A). Mapping Dnmt3a binding sites to regions flanking transcription start sites (TSSs) revealed two categories of Dnmt3a targets (Fig. 2A). The first group (*n* = 2449 targets) was characterized by the presence of CpG islands, which generally harbor high levels of trimethylation of lysine 4 on histone H3 (H3K4me3), a histone mark indicative of transcriptionally permissive chromatin states, within their proximal promoter regions (hereafter referred to as CpG-rich/H3K4me3-high targets) (Fig. 2A and fig. S8, A to C). On these targets, Dnmt3a was generally excluded from H3K4me3-high, CpG-rich proximal promoters, but was enriched in inter- and intragenic regions flanking CpG islands (black in Fig. 2B), possibly because of the inhibitory effect of H3K4 methylation on Dnmt3a binding to chromatin (14). The other group of Dnmt3a targets (*n* = 1096) was associated with CpG-poor promoters and low levels of H3K4me3 (Fig. 2A and fig. S8, A to C). Dnmt3a binding sites on these H3K4me3-low targets frequently overlapped with proximal promoters (orange in Fig. 2B). Subsequent genome-wide methylated DNA immunoprecipitation assays (MeDIP-chip) revealed that Dnmt3a-dependent DNA methylation was restricted to Dnmt3a binding sites (Fig. 2C and fig. S9B). Locus-specific methylation assays (MeDIP-qPCR, bisulphite sequencing, or mass spectrometry) further demonstrated that, on H3K4me3-high targets (for instance, *Dlx2*, *Gbx2*), Dnmt3a-dependent DNA methylation was present in regions encompassing proximal promoters, but not within core promoter regions (Fig. 2, C and D, and figs. S7; S10, A and B; and S11, B and C). In contrast, Dnmt3a-mediated methylation was enriched at proximal promoters of H3K4me3-low

¹Department of Molecular and Medical Pharmacology, University of California Los Angeles (UCLA), Los Angeles, CA 90095, USA. ²Department of Psychiatry and Biobehavioral Sciences, Intellectual Development and Disabilities Research Center at Semel Institute for Neuroscience, UCLA, Los Angeles, CA 90095, USA. ³Molecular Biology Institute, UCLA School of Medicine, Los Angeles, CA 90095, USA. ⁴Laboratory of Regulation of Neuronal Development, Institute for Protein Research, Osaka University, Suita, Osaka, 565-0871, Japan. ⁵Novartis Institutes for Biomedical Research, Cambridge, MA 02139, USA. ⁶Howard Hughes Medical Institute, Department of Biochemistry and Biophysics, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.

*To whom correspondence should be addressed. E-mail: ysun@mednet.ucla.edu (Y.E.S.); haowu7@gmail.com (H.W.)

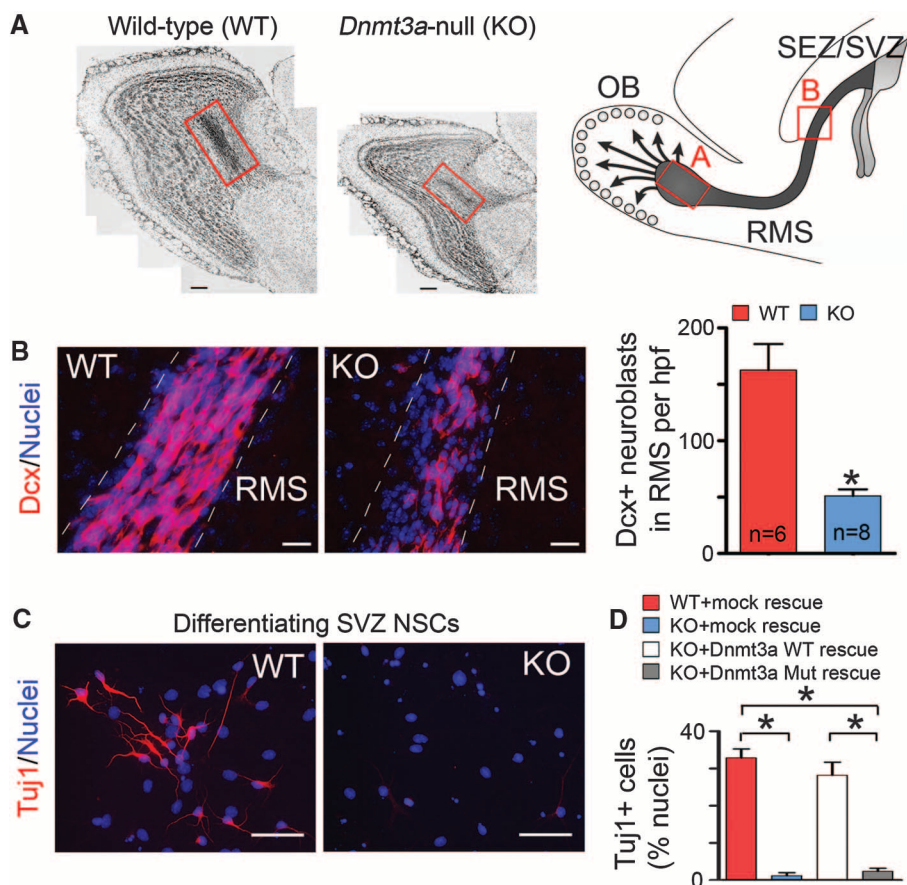


Fig. 1. Essential roles of Dnmt3a in postnatal neurogenesis. **(A)** Cell nuclei staining of olfactory bulb (OB) in WT and KO mice at postnatal day (P) 24. The schematic on the right outlines the postnatal neurogenesis from SEZ/SVZ NSCs, and the red boxes denote the portion of the rostral migratory stream (RMS) with newborn neurons entering the OB. Note that both the OB size and the number of newborn neurons in KO mice (red box) are reduced. Arrows indicate radially migrating immature neurons in the postnatal OB. Scale bars, 200 μ m. **(B)** Immunohistochemistry and quantification of Dcx+ neuroblasts in WT and KO RMS (P21 to P24). Hpf, high-power field; *n*, number of fields from three pairs of littermate mice. Error bars indicate SEM ($*P < 0.01$). Scale bars, 50 μ m. **(C)** Immunocytochemistry of the immature neuronal marker Tuj1 in differentiating WT and KO SEZ/SVZ cultures. Scale bars, 50 μ m. **(D)** Quantification of Tuj1+ neurons in differentiating WT, KO, and rescued NSC cultures. Error bars indicate SEM ($*P < 0.01$, $n = 10$ to 15).

targets (e.g., *Gfap*) (Fig. 2, C and D, and fig. S7C). Although DNA methylation changes have been reported after long-term cell culture (15), short-term in vitro culturing in our experiments did not significantly alter promoter DNA methylation levels, either globally or on Dnmt3a targets (fig. S10, B and C). Collectively, these data indicate that Dnmt3a may occupy and methylate defined genomic regions associated with both transcriptionally active and inactive genes in postnatal NSCs.

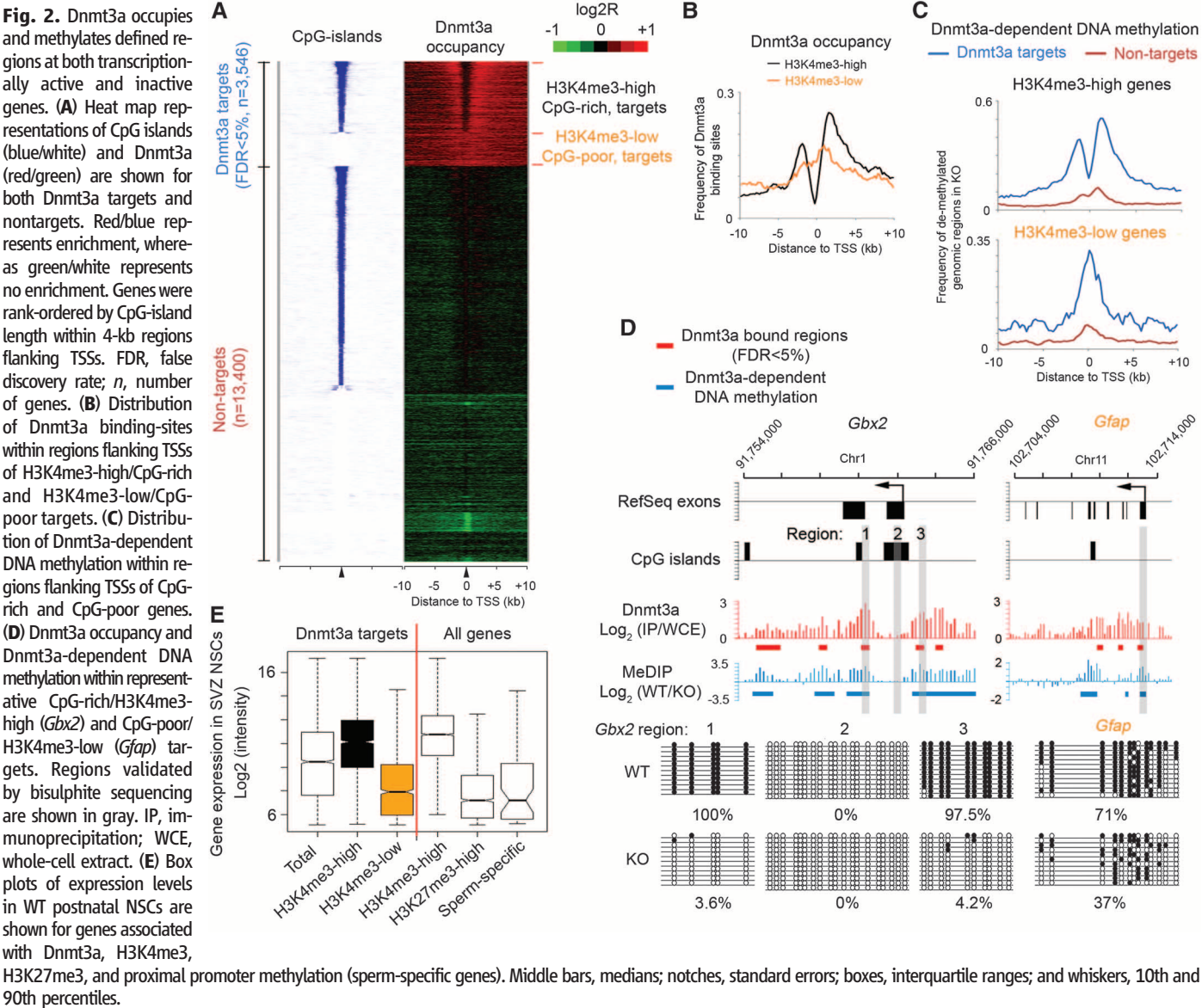
To gain insights into the biological function of Dnmt3a targets, we performed gene ontology analysis with Dnmt3a targets. Genes functionally related to nervous system development ($P = 1.5 \times 10^{-30}$, hypergeometric test) and neurogenesis ($P = 6.5 \times 10^{-19}$) were significantly enriched within H3K4me3-high Dnmt3a targets, whereas genes involved in development of non-neuronal lineage were enriched in H3K4me3-low Dnmt3a targets (fig. S8D). H3K4me3-high targets were transcribed at medium-to-high levels in postnatal NSCs, signif-

icantly higher than expression levels of H3K4me3-low targets, or genes marked by an inactive histone modification H3K27me3 or a group of sperm-specific genes with proximal promoter DNA methylation (Fig. 2E).

To establish a direct link between Dnmt3a binding and transcriptional regulation, we performed gene-expression profiling and identified a total of 2275 genes (1253 up-regulated and 1022 down-regulated in *Dnmt3a*-null mice) that are differentially expressed between undifferentiated WT and KO NSCs. Dnmt3a binding sites were detected within 10-kb up- and downstream of the TSSs of 942 differentially expressed transcripts (41.4% of 2275 genes, $P = 1.36 \times 10^{-122}$, Fisher's exact test) (Fig. 3A), indicating that a significant fraction of Dnmt3a-dependent expression changes represent a direct effect of Dnmt3a binding. Although DNA methylation has been primarily associated with repression, Dnmt3a targets were significantly enriched in both up- and down-

regulated genes in KO NSCs (536 up-regulated, $P = 3.3 \times 10^{-71}$; 406 down-regulated, $P = 1.9 \times 10^{-43}$). Notably, genes with known roles in postnatal neurogenesis (for example, *Dlx2*, *Sp8*, and *Neurog2*) were among the down-regulated Dnmt3a targets in KO NSCs (fig. S11A) (16, 17). In contrast, several Dnmt3a targets involved in astroglial and oligodendroglial differentiation (e.g., *Sparcl1*, *Nkx2-2*) were up-regulated in KO cultures. Gene-expression analyses with differentiating NSCs also supported the conclusion that Dnmt3a promotes transcription of neurogenic targets while repressing glial differentiation genes (table S1 and supporting online material text). Further in vivo analysis revealed fewer Dlx2+ and Sp8+ progenitors/neuroblasts and their subtype-specific interneuron progenies in KO mice (fig. S12, A to E), and re-expression of *Dlx2* in KO cells substantially rescued the neurogenesis defect (fig. S12F). Taken together, these results indicate that Dnmt3a is not only involved in transcriptional repression but also required by postnatal NSCs to promote expression of many direct targets, including key neurogenic factors, thereby contributing to postnatal neurogenesis.

The percentage of H3K4me3-high promoters in down-regulated targets (65.4% of 406 genes) was significantly higher than that expected by chance ($P = 7.7 \times 10^{-8}$), and DNA demethylation was predominantly detected outside proximal promoters of these down-regulated targets in KO NSCs (Fig. 3A and fig. S11, B and C). In contrast, DNA demethylation on up-regulated targets in KO NSCs (red in Fig. 3B) was more enriched at proximal promoters compared with down-regulated targets (green compared with red in Fig. 3B; $P = 0.0018$) or targets without expression changes (blue in Fig. 3B; $P = 6.8 \times 10^{-9}$). Although proximal promoter DNA methylation may repress transcription by antagonizing active histone marks (Fig. 3A) (15, 18, 19), growing evidence suggests that non-promoter DNA methylation positively correlates with transcription of tissue-specific genes or genes on the active X chromosome (2, 20). DNA methylation also inversely correlates with the presence of H3K27me3 in human cancer cell lines or on the transcriptional regulatory region for the imprinting locus *Rasgrf1* (21, 22). Therefore, we hypothesized that Dnmt3a-dependent nonpromoter DNA methylation may facilitate transcription by antagonizing H3K27me3. To test this idea, we examined genome-wide H3K27me3 occupancy in WT and KO NSCs. Our analysis revealed that *Dnmt3a*-deficiency resulted in an increase in H3K27me3 levels across large genomic regions associated with many down-regulated targets (green in Fig. 3B and fig. S11, B and C), whereas the increase in H3K27me3 levels in KO NSCs was observed less often on up-regulated targets (red in Fig. 3B; $P = 1.8 \times 10^{-20}$) or targets without changes in expression (blue in Fig. 3B; $P = 1.6 \times 10^{-28}$). Acute ablation of Dnmt3a also led to a significant increase in H3K27me3 levels on down-regulated targets, suggesting that the antagonism between Dnmt3a and H3K27me3 is unlikely due to



nonspecific compensation after long-term *Dnmt3a* deletion (fig. S5D). Thus, Dnmt3a-dependent non-promoter DNA methylation may facilitate the expression of many H3K4me3-high targets by reducing H3K27me3 levels.

Because *Dnmt3a* deficiency did not change the overall levels of H3K27me3 or Polycomb repression complex 2 (PRC2) that catalyzes trimethylation of H3K27 (fig. S13, A and B), we considered a model in which Dnmt3a functioned to antagonize PRC2 binding to chromatin. ChIP-chip analysis of Suz12 and Ezh2, two core subunits of PRC2, revealed that *Dnmt3a* deficiency led to a significant increase in Suz12 occupancy on Dnmt3a targets with a marked increase in H3K27me3 in KO NSCs ($P < 2.2 \times 10^{-16}$); similar results were obtained for Ezh2 (Fig. 4A and fig. S11, D and E). The increase of PRC2/H3K27me3 levels on these Dnmt3a targets was specific, as other PRC2 targets that were not targeted by Dnmt3a (e.g., *Hoxb9* in Fig. 4A) were associated with similar levels of PRC2/H3K27me3 in WT and KO NSCs. To

evaluate whether Dnmt3a enzymatic activity is required for opposing PRC2 binding, we analyzed *Dnmt3a*-null NSCs rescued with physiological levels of WT or catalytically inactive mutant Dnmt3a. In rescued KO NSCs, both WT and mutant Dnmt3a re-occupied Dnmt3a targets (fig. S14A), but only WT Dnmt3a restored DNA methylation levels (fig. S14, B and C). WT, but not mutant Dnmt3a, reversed aberrantly elevated levels of PRC2/H3K27me3 on down-regulated Dnmt3a targets in KO NSCs (Fig. 4B), indicating that Dnmt3a-dependent DNA methylation, but not Dnmt3a binding, is incompatible with PRC2 occupancy and may directly antagonize the PRC2 binding. Therefore, we tested recruitment of PRC2 complexes to in vitro DNA-methylated chromatin arrays consisting of recombinant *Xenopus* octamers (23, 24), which are free of any other epigenetic marks existing on endogenous chromatin that may also regulate PRC2 recruitment (fig. S15). This analysis indicated that DNA methylation alone partially inhibited PRC2 binding to

chromatin arrays (Fig. 4C). Furthermore, PRC2 deficiency in *Dnmt3a*-null NSCs could substantially restore the expression of neurogenic targets and rescue neuronal differentiation defects (Fig. 4D and fig. S13, C and D). Dnmt3a-dependent DNA methylation may, therefore, down-regulate H3K27me3 levels on transcriptionally active targets by antagonizing binding of PRC2 to chromatin.

Together, these results suggest that the DNA methyltransferase Dnmt3a not only mediates repression in self-renewing postnatal NSCs by methylating proximal promoters, but also promotes transcription of targets, including neurogenic genes, by antagonizing Polycomb repression through nonproximal promoter methylation (fig. S16). The positive correlation between nonpromoter DNA methylation and transcription on Dnmt3a targets is also observed during mouse ES cell differentiation into neural progenitors, as well as in vivo analysis of multiple adult tissues, suggesting that this finding may be generally applicable to other

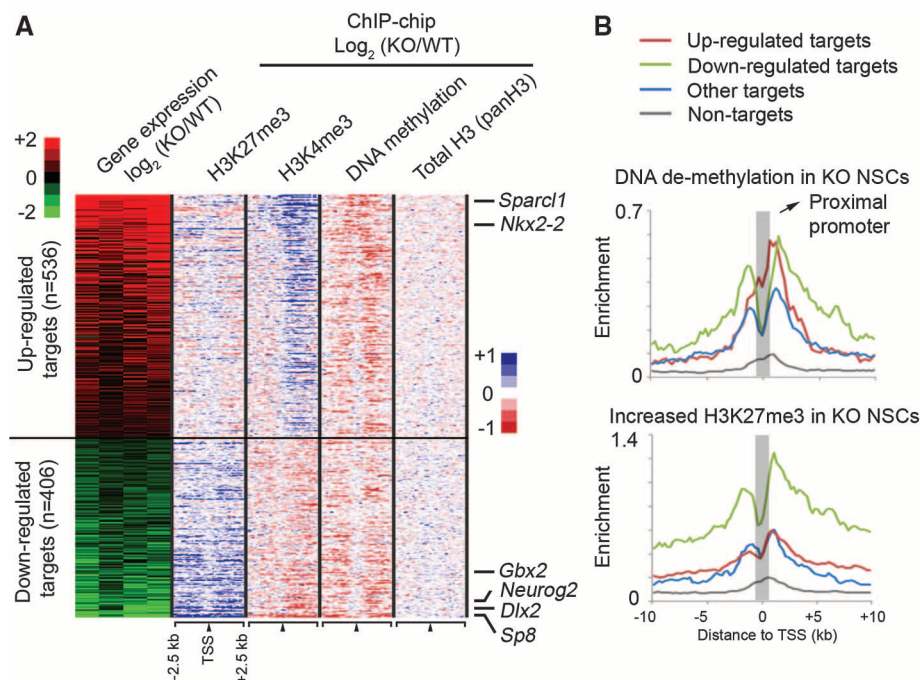
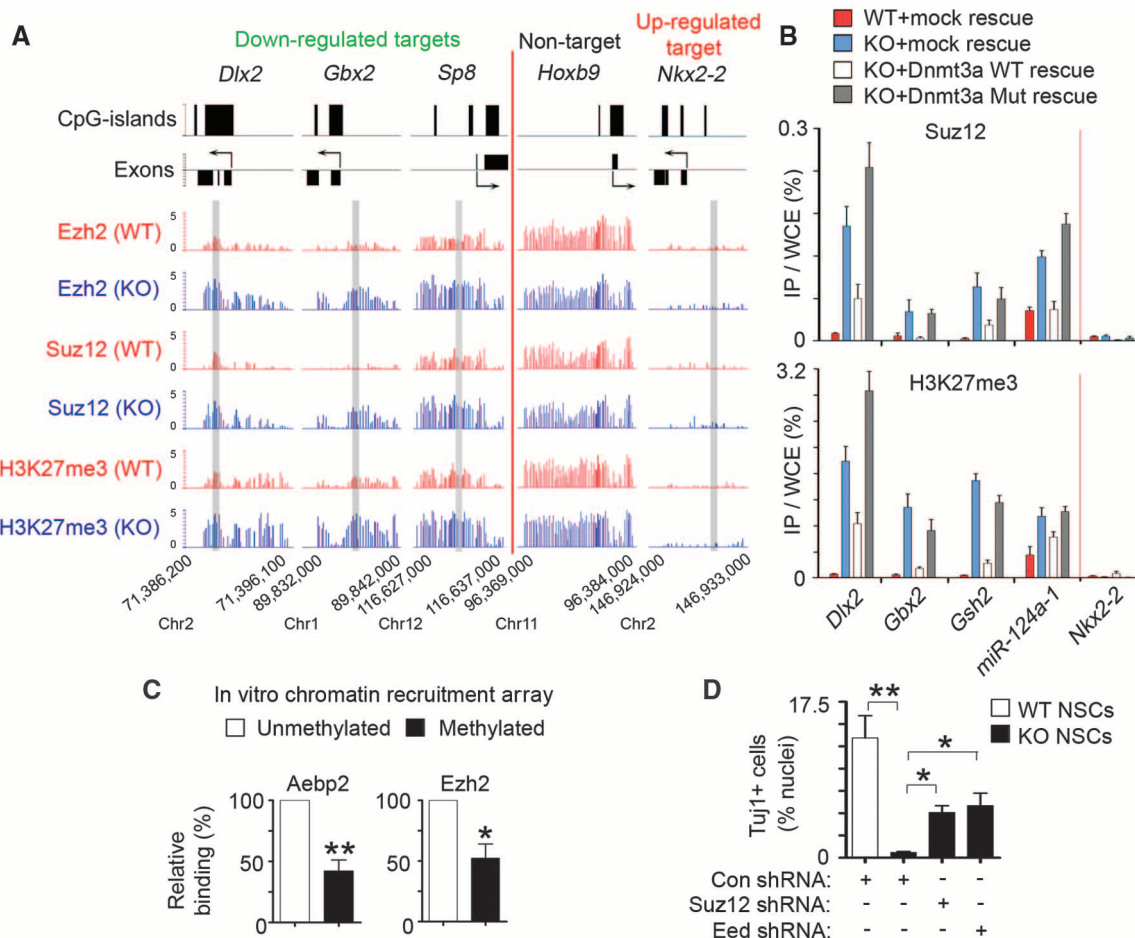


Fig. 3. Dnmt3a promotes transcription through nonpromoter methylation. **(A)** Heat map representations of relative changes in expression (from four experiments), DNA methylation, H3K4me3, H3K27me3, and total histone H3 levels between KO and WT NSCs are shown for differentially expressed Dnmt3a targets, which are ranked by changes in expression between KO and WT NSCs. *n*, number of targets. **(B)** Distributions of DNA demethylation and H3K27me3 increase in KO NSCs are shown for up-regulated targets (red), down-regulated targets (green), targets without expression changes (blue), and nontargets (gray).

Fig. 4. Dnmt3a promotes transcription by antagonizing Polycomb repression. **(A)** Ezh2, Suz12, and H3K27me3 occupancy at representative genes in WT and KO NSCs. Regions analyzed by ChIP-qPCR (in fig. S11E) are shaded in gray. **(B)** Quantitative PCR analysis of Suz12 and H3K27me3 at representative targets in WT, KO, and rescued NSCs. **(C)** Relative binding of PRC2 subunits on methylated or unmethylated chromatin arrays was quantified by densitometry (**P* = 0.027, *n* = 4 experiments; ***P* = 0.0013, *n* = 6). **(D)** Quantification of Tuj1+ neurons in control and PRC2-deficient differentiating NSCs. Error bars indicate SEM of randomly selected fields from two independent cultures (**P* < 0.05; ***P* < 0.01).



developmental processes (figs. S17 and S18). Embryonic neurogenesis is largely intact in *Dnmt3a*-null mice (10), perhaps because embryonic neuroblasts are primarily generated from neuroepithelial progenitors where Dnmt3b is still highly expressed (13). Furthermore, Polycomb repression can also be inhibited by H3K27me3 demethylase (e.g., Jmjd3), which may be recruited by H3K4 methyltransferases Mll to promoters of neurogenic factors during embryonic and/or postnatal neurogenesis (16, 25). Non-proximal promoter DNA methylation established by Dnmt3a may, therefore, represent an additional layer of epigenetic regulation of Polycomb repression in somatic cells. Although PRC2 may directly interact with Dnmt proteins (26, 27), it seems that DNA methyltransferases (for instance, catalytically inactive Dnmt3a) may coexist with high levels of PRC2 occupancy at the same genomic loci (27), only in the absence of de novo DNA methylation. Thus, Polycomb proteins may contribute to the initial recruitment of Dnmt, the accumulation of which leads to local de novo DNA methylation, thereby dampening Polycomb repression. Given that both DNA methylation and Polycomb pathways are indispensable for normal development and are implicated in diseases, including neurological disorders and cancer (21, 28), it will be of interest to fully elucidate mechanisms by which these two epigenetic machineries are targeted to specific genomic loci and are cross-regulated.

References and Notes

- R. Jaenisch, A. Bird, *Nat. Genet.* **33**, 245 (2003).
- M. M. Suzuki, A. Bird, *Nat. Rev. Genet.* **9**, 465 (2008).
- R. Lister *et al.*, *Nature* **462**, 315 (2009).
- R. A. Irizarry *et al.*, *Nat. Genet.* **41**, 178 (2009).
- M. Okano, D. W. Bell, D. A. Haber, E. Li, *Cell* **99**, 247 (1999).
- E. Li, T. H. Bestor, R. Jaenisch, *Cell* **69**, 915 (1992).
- S. Nguyen, K. Meletis, D. Fu, S. Jhaveri, R. Jaenisch, *Dev. Dyn.* **236**, 1663 (2007).
- M. Chahrour, H. Y. Zoghbi, *Neuron* **56**, 422 (2007).
- J. Feng, H. Chang, E. Li, G. Fan, *J. Neurosci. Res.* **79**, 734 (2005).
- H. Suh, W. Deng, F. H. Gage, *Annu. Rev. Cell Dev. Biol.* **25**, 253 (2009).
- Materials and methods are available as supporting material on Science Online.
- M. Kaneda *et al.*, *Nature* **429**, 900 (2004).
- T. Chen, N. Tsujimoto, E. Li, *Mol. Cell. Biol.* **24**, 9048 (2004).
- J. Otani *et al.*, *EMBO Rep.* **10**, 1235 (2009).
- A. Meissner *et al.*, *Nature* **454**, 766 (2008).
- D. A. Lim *et al.*, *Nature* **458**, 529 (2009).
- R. R. Wacław *et al.*, *Neuron* **49**, 503 (2006).
- S. K. Ooi *et al.*, *Nature* **448**, 714 (2007).
- M. Weber *et al.*, *Nat. Genet.* **39**, 457 (2007).
- R. Straussman *et al.*, *Nat. Struct. Mol. Biol.* **16**, 564 (2009).
- E. N. Gal-Yam *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **105**, 12979 (2008).
- A. M. Lindroth *et al.*, *PLoS Genet.* **4**, e1000145 (2008).
- R. Cao *et al.*, *Science* **298**, 1039 (2002); published online 26 September 2002 (10.1126/science.1076997).
- A. Smallwood, P. O. Estève, S. Pradhan, M. Carey, *Genes Dev.* **21**, 1169 (2007).
- K. Jepsen *et al.*, *Nature* **450**, 415 (2007).
- E. Viré *et al.*, *Nature* **439**, 871 (2006).
- M. Rush *et al.*, *Epigenetics* **4**, 404 (2009).
- K. D. Robertson, *Nat. Rev. Genet.* **6**, 597 (2005).
- We thank M. Carey (UCLA) for technical assistance on in vitro chromatin recruitment assays and J. Rubenstein (University of California San Francisco) for providing the pCAG-Dlx2 expression plasmid. This work was supported by NIH RO1 grants to Y.E.S. All microarray data are deposited in Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) under accession number GSE22476.

Supporting Online Material

www.sciencemag.org/cgi/content/full/329/5990/444/DC1

Materials and Methods

SOM Text

Figs. S1 to S18

Tables S1 and S2

References

5 April 2010; accepted 3 June 2010

10.1126/science.1190485

Functional Modules and Structural Basis of Conformational Coupling in Mitochondrial Complex I

Carola Hunte,^{1,2,3*} Volker Zickermann,^{4*} Ulrich Brandt^{4†}

Proton-pumping respiratory complex I is one of the largest and most complicated membrane protein complexes. Its function is critical for efficient energy supply in aerobic cells, and malfunctions are implicated in many neurodegenerative disorders. Here, we report an x-ray crystallographic analysis of mitochondrial complex I. The positions of all iron-sulfur clusters relative to the membrane arm were determined in the complete enzyme complex. The ubiquinone reduction site resides close to 30 angstroms above the membrane domain. The arrangement of functional modules suggests conformational coupling of redox chemistry with proton pumping and essentially excludes direct mechanisms. We suggest that a ~60-angstrom-long helical transmission element is critical for transducing conformational energy to proton-pumping elements in the distal module of the membrane arm.

Mitochondrial complex I [proton-pumping NADH (the reduced form of nicotinamide adenine dinucleotide):ubiquinone oxidoreductase] is a macromolecular membrane complex of close to one million daltons with a central function in energy metabolism (*1*). It is the entry point for electrons from NADH into the respiratory chain, and it couples electron transfer to ubiquinone with vectorial proton pumping across the inner mitochondrial membrane. With a stoichiometry of four protons to two electrons ($4\text{H}^+/2\text{e}^-$), it contributes about 40% of the proton motive force that drives adenosine 5'-triphosphate (ATP) syn-

thesis by ATP synthase (*2, 3*). Complex I is a source for deleterious reactive oxygen species (ROS) (*4, 5*) and has been implicated in the pathogenesis of a variety of neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Leigh syndrome (*6, 7*). Moreover, ROS generation by complex I may contribute to aging processes (*8*).

Despite the important physiological role of complex I, its molecular mechanism of energy conversion is essentially unknown. Progress in understanding complex I function has been severely limited by the lack of detailed structural information. Electron microscopy (EM) shows an L-shaped overall architecture with a membrane arm and a hydrophilic peripheral arm that protrudes into the mitochondrial matrix (*9, 10*) (Fig. 1A). The x-ray structure of the peripheral arm from a bacterial complex I was determined (*11, 12*).

The peripheral arm consists of two functional modules (*1*) and comprises all redox active cofactors (*11*). The N module contains the NADH oxidation site with one flavin mononucleotide (FMN) molecule as the primary electron acceptor, whereas the Q module contains the ubiquinone reduction site (Fig. 1A). Fast electron transfer between the two catalytic sites (*13*) is mediated by

a chain of seven iron-sulfur clusters. An additional iron-sulfur cluster localized near the FMN cofactor may serve a special function in protection against ROS formation (*13–15*). The membrane arm or P module harbors the proton-pumping machinery (*16*), presumably involving the three subunits—ND2, ND4, and ND5—that are homologous to sodium-proton antiporters (*17*).

Complex I is substituted by alternative enzymes in the widely used model organism *Saccharomyces cerevisiae*. Therefore, we have established the aerobic yeast *Yarrowia lipolytica* as a genetic model system to study eukaryotic complex I (*18*). In the complex from this source, 40 different subunits were identified with a total mass of 946.5 kD (*19, 20*). Fourteen central subunits are highly conserved among eukaryotes and prokaryotes (*1*). They form the structural core of the two arms of the complex and are essential for its bioenergetic functions. Twenty-six accessory subunits that are not found in prokaryotes are arranged around this core and presumably function in assembly, stabilization, regulation, and additional metabolic pathways that are not directly linked to energy conservation.

Here we report the x-ray crystallographic analysis of complete *Y. lipolytica* complex I (*21*). Phase information was obtained with anomalous data of a heavy atom cluster derivative and with native data (table S1). The molecule is L-shaped, with an angle between the two arms of about 100° (Fig. 1B). The membrane arm has a length of ~180 Å, and the distance between the tip of the peripheral arm and the bottom of the membrane arm is ~190 Å. At a resolution of 6.3 Å, many secondary structure elements and especially α -helical transmembrane segments were clearly recognized in the electron density.

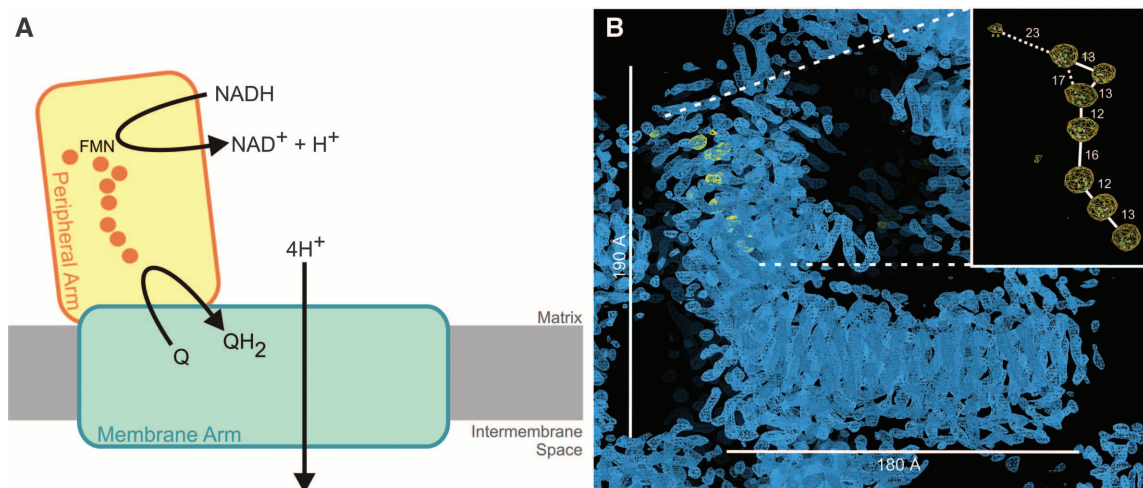
The positions of all eight iron-sulfur cofactors were localized in the peripheral arm by anomalous Fourier analysis (Fig. 1B and table S1). The distances between the iron-sulfur clusters are similar to those reported for the hydrophilic fragment of bacterial complex I (*15*). Seven of these clusters constitute a continuous electron wire between the catalytic sites. Manual superimposition with the partial bacterial structure based on the

¹Institute for Biochemistry and Molecular Biology, Centre for Biological Signalling Studies (BIOSS), University of Freiburg, D-79104 Freiburg, Germany. ²Institute of Membrane and Systems Biology, University of Leeds, Leeds LS2 9JT, UK. ³Department of Molecular Membrane Biology, Max Planck Institute of Biophysics, D-60438 Frankfurt am Main, Germany. ⁴Molecular Bioenergetics Group, Medical School, Cluster of Excellence Frankfurt "Macromolecular Complexes," Center for Membrane Proteomics, Goethe-University, D-60596 Frankfurt am Main, Germany.

*These authors contributed equally to this work.

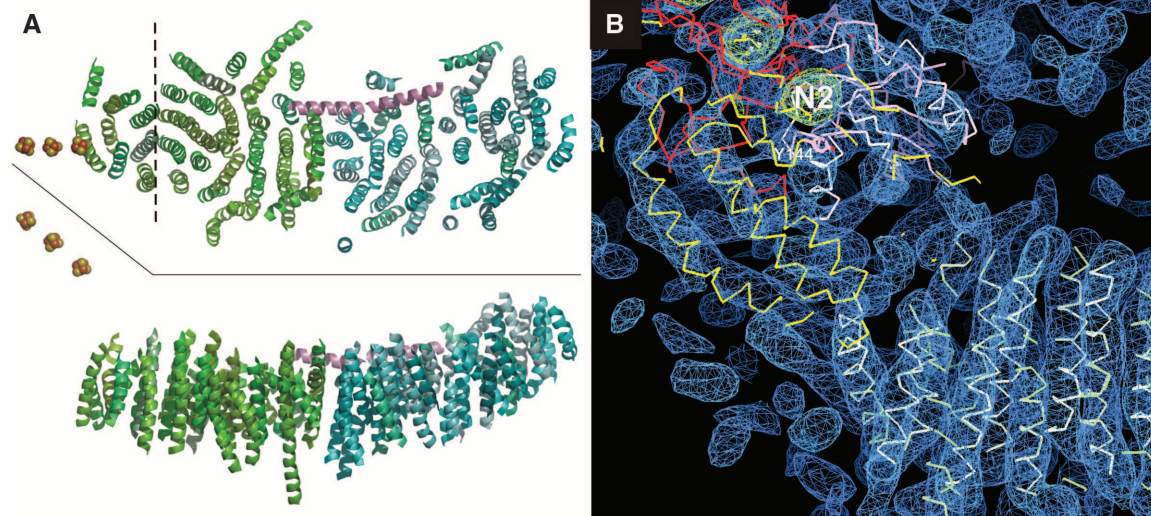
†To whom correspondence should be addressed. E-mail: brandt@zbc.kgu.de

Fig. 1. Overall shape and iron-sulfur cluster positions. **(A)** Schematic depiction of overall organization and function of mitochondrial complex I. FMN and a chain of iron-sulfur clusters (orange spheres) in the peripheral arm connect the NADH oxidation site with the ubiquinone (Q) reduction site. The redox reaction is linked to the translocation of four protons across the inner mitochondrial membrane. **(B)** Well-defined electron density corresponding to one molecule of L-shaped complex I shown in side view with the membrane arm displayed horizontally. The anomalous Fourier map contoured at 4.5σ , indicating the positions of eight iron-sulfur clusters of the peripheral arm, is displayed in yellow and shown enlarged and slightly turned for better visualization of all



clusters in the insert. Center-to-center distances are given in angstroms. Solid white lines indicate that these distances are short enough for physiological electron transfer; otherwise, dashed lines are used. The anomalous map is overlaid with cluster positions from the bacterial partial structure.

Fig. 2. Transmembrane segments and interface of the membrane arm and the peripheral arm. **(A)** Top view from matrix side and side view of the α -helical model of the transmembrane segments (upper and lower panel, respectively) fitted with helices arranged in a proximal (green) and a distal (cyan) domain. The border of the docking area with the peripheral arm is indicated by a dashed line (top). The three iron-sulfur clusters of the Q module are shown in space-filled representation. An extended helical element (magenta) may be critical for energy transmission within the membrane arm. **(B)** Side view of electron density with transmembrane segments including their helical model and the superimposed structure of the bacterial peripheral arm fragment (3IAM). Yellow, 49-kD subunit; pink, PSST subunit; red, TYKY subunit;



green, anomalous Fourier map indicating positions of iron-sulfur clusters. The vertical distance from iron-sulfur cluster N2, which is the immediate electron donor to ubiquinone, to the membrane surface is $\sim 30 \text{ \AA}$. The conserved tyrosine (Y144) implicated in binding the ubiquinone head group is shown in light purple.

well-defined positions of the iron-sulfur clusters and prominent secondary structure elements revealed that two parts of the peripheral arm form rigid bodies corresponding to the Q and N modules. Compared with the bacterial fragment, the Q and N modules are opened 3° wider and turned slightly, relative to each other. This reorientation does not affect the distances for electron transfer between the two parts of complex I.

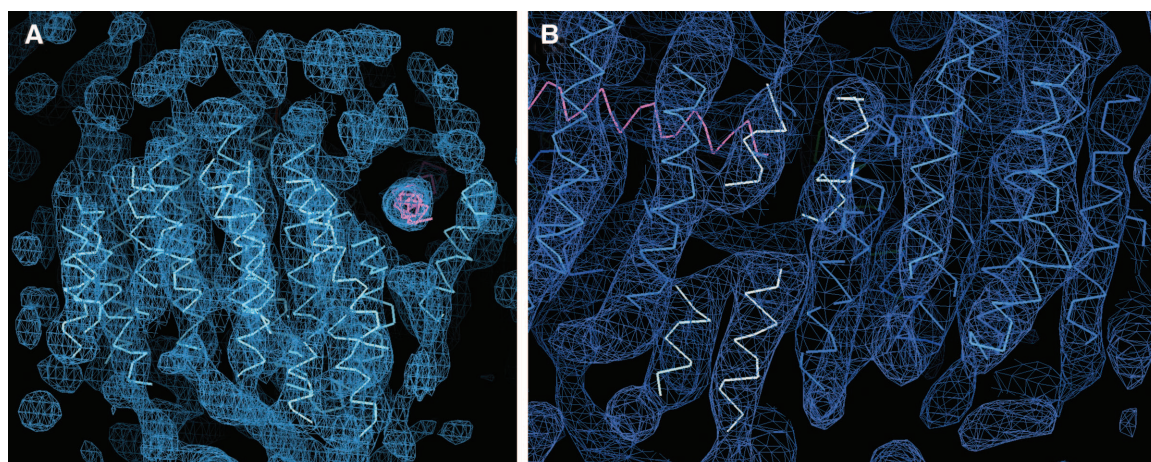
For the P module, prediction algorithms and mapping experiments suggest the presence of 17 membrane-integral subunits with more than 70 transmembrane segments (16). We modeled 71 α -helical transmembrane segments of different lengths, tilts, and curvatures into continuous elongated electron density features (Fig. 2A). At least

two of the latter appear interrupted in the middle of the membrane core, which is suggestive of discontinuous helices that are a hallmark of sodium-proton antiporters and other ion-translocating proteins (22). This finding is reminiscent of the homology of subunits ND2, ND4, and ND5 to subunits of bacterial sodium-proton antiporters (17). Overall, the side view of the transmembrane segments (Fig. 2A, bottom) reveals a curved shape, with the concave side facing the mitochondrial matrix. This appearance of the membrane arm was also observed for bacterial complex I by EM (10, 23). The extrinsic protein portion on the matrix side largely consists of the peripheral arm and a protrusion at the distal end (Fig. 1B). In contrast, the intermembrane side is characterized by a prominent

layer of electron density that is spread over the entire membrane arm. The layer includes well-defined elongated density features that probably represent long surface helices extending parallel to the long axis of the membrane arm. The membrane arm shows two entities of about equal size dividing the P module into a proximal and distal domain (Fig. 2A). They are connected by a narrow, centrally located interface occupying only about half of the width of the membrane arm. The analysis of defined subcomplexes had already suggested a modular architecture of the membrane (24), and the proposed proton-translocating subunits ND4 and ND5 can be assigned to the distal P domain.

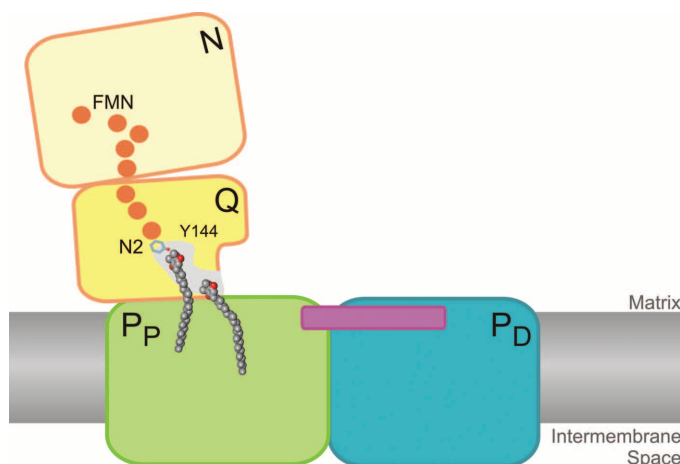
The driving force for proton translocation must be generated in the peripheral arm from the

Fig. 3. Transmission element connecting the P domains, a putative central component of the proton-pumping machinery. The transmission element, seen as elongated density feature close to the matrix-exposed surface, is fitted with a helical structure (magenta). Connecting the proximal with the distal P domain, this helix may drive proton pumping by conferring conformational coupling. The helical model of the transmembrane segments is shown in blue. (A) View in parallel to the transmission element from the distal P domain along the long axis of the membrane arm. (B) Partial side view of the distal P domain shows electron



density features that reside near the end of the transmission element and resemble discontinuous helix arrangements (light blue) that are a hallmark of ion-translocating proteins.

Fig. 4. Schematic model of the four functional modules of complex I. A chain of seven iron-sulfur clusters (orange spheres) leads from the N module (light yellow) with FMN as the primary electron acceptor to the ubiquinone reduction site in the Q module (yellow) involving cluster N2 and tyrosine-144. The position of N2 is close to 30 Å above the membrane surface. With the ubiquinone headgroup (space-fill representation) in functional distance to the electron donor at the end of the extended binding cavity (light gray), the hydrophobic isoprenoid side chain will be about half-way out of the core membrane region. This suggests the presence of a hydrophobic access path and excludes direct coupling between redox chemistry in the N and Q modules and vectorial proton translocation by subunits of the proximal (P_P, green) and distal (P_D, cyan) domains of the P module. An extended transmission element (magenta) forms a bridge across the two domains.



energy released during electron transfer from NADH to ubiquinone. Several lines of evidence suggest that it is the redox chemistry of ubiquinone that triggers the proton-pumping machinery (16, 25, 26). Thus, the spatial arrangement of the Q and P modules, and the transmission of energy between them, is fundamental to redox-linked proton translocation by complex I.

Critical residues for interaction with ubiquinone and inhibitors are found at the interface of the highly conserved 49-kD and PSST subunits (for complex I subunits, the nomenclature of the bovine heart enzyme is used) of the Q module (27–29) (fig. S1 and table S1) that are homologous to the large and small subunit of [NiFe] hydrogenase and form the ubiquinone-reducing catalytic core of complex I (1). The three iron-sulfur clusters of the Q module provide a precise anchoring point for the superimposition of the bacterial partial structure with respect to the membrane arm. The

49-kD subunit contains a characteristic four-helix bundle that is seen in the experimental electron density map (Fig. 2B). The three iron-sulfur clusters of the Q module line up at an angle of 130 to 140° with respect to the membrane arm (Fig. 1B). In the top view, the vertical projection of this array is centrally oriented in parallel to the long axis of the membrane arm. The 49-kD and PSST subunits dock on the proximal end of the membrane arm (Fig. 2A, top). The main part of the docking site is covered by the 49-kD subunit with its conserved N-terminal β sheet that is oriented toward but outside of the membrane arm surface. A smaller part is composed of parts of the PSST subunit. In the membrane arm, the docking site is bordered by four long, straight, and densely arranged electron density features, indicating a row of four hardly tilted transmembrane helices (Fig. 2A). The contact site has a more open appearance toward subunit PSST, and we suggest

that this area provides access for ubiquinone from the phospholipid bilayer.

The last iron-sulfur cluster in the chain, called N2, is the immediate electron donor for ubiquinone. A funnel-like cavity leading from the N-terminal β sheet of the 49-kD subunit toward tyrosine-144, (tyrosine-87 in *Thermus thermophilus*), which is in the immediate vicinity of cluster N2, has been mapped by site-directed mutagenesis (27, 28). It has been shown by detailed structure/function analysis that ubiquinone binds directly to this fully conserved tyrosine (29). Measured perpendicular to the membrane plane, cluster N2 resides ~ 30 Å above the surface of the transmembrane core (Fig. 2B). The distance from the opening of the ubiquinone-binding pocket at the surface of the membrane arm to the electron donor site is ~ 35 Å. Considering that the binding site for ubiquinone must be at a distance allowing electron transfer of cluster N2, the substrate must diffuse ~ 25 Å out from the hydrophobic core of the membrane to position the functional head group in the active site. This distance accounts for much of the extremely hydrophobic 40-Å-long side chain of nine isoprenoid units in ubiquinone that may slide over a hydrophobic ramp as previously proposed (30, 31).

This arrangement of the peripheral arm has far-reaching implications for the catalytic mechanism of complex I and is similar to one of the possible orientations (fit 2) determined in a previous study by using single-particle EM (31) but differs from that suggested by Sazanov and co-workers (11, 23). The position of the ubiquinone reduction site separate from the transmembrane domain of complex I seems to rule out any mechanism that would use redox-linked proton uptake and release directly for vectorial proton translocation. Rather, the energy released in the redox reactions is transmitted to the proton-pumping machinery via long-range conformational changes (1, 16, 32). Considering that subunits ND4 and ND5 at the distal end of the membrane arm are

among the prime candidates to harbor proton-translocation sites, distances for energy-transfer reactions of more than 100 Å have to be envisaged. A continuous electron density most likely corresponding to an α helix is laterally associated with the membrane arm embedded in the transmembrane segments near the matrix-facing surface (Fig. 3). With a length of 60 Å oriented in parallel to the long axis of the membrane arm, it extends from the end of the proximal domain about half-way into the distal domain (Fig. 2A). At the proximal end, it is connected by continuous electron density to one of the transmembrane segments in a nearly orthogonal orientation. Strikingly, on the P_D domain side, it terminates close to electron density features resembling a discontinuous helix arrangement, suggesting the presence of a proton-translocation unit (Fig. 3B) (22). We propose that this observed connection between the P domains is a critical transmission element within the proton-pumping machinery of complex I.

The modular architecture of complex I as revealed by crystallographic analysis of the complete mitochondrial enzyme is summarized in Fig. 4. In a bipartite functional organization, conformational energy is generated by the redox chemistry occurring in the N and Q modules of the peripheral arm and is transmitted to the two proton-pumping modules of the membrane arm, which are connected by a helical transmission element.

References and Notes

1. U. Brandt, *Annu. Rev. Biochem.* **75**, 69 (2006).
2. M. K. F. Wikström, *FEBS Lett.* **169**, 300 (1984).
3. A. Galkin, S. Dröse, U. Brandt, *Biochim. Biophys. Acta* **1757**, 1575 (2006).
4. A. Galkin, U. Brandt, *J. Biol. Chem.* **280**, 30129 (2005).
5. L. Kussmaul, J. Hirst, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 7607 (2006).
6. M. T. Lin, M. F. Beal, *Nature* **443**, 787 (2006).
7. V. Rhein *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 20057 (2009).
8. H. Fukui, C. T. Moraes, *Trends Neurosci.* **31**, 251 (2008).
9. M. Radermacher *et al.*, *J. Struct. Biol.* **154**, 269 (2006).
10. T. Clason *et al.*, *J. Struct. Biol.* **169**, 81 (2010).
11. L. A. Sazanov, P. Hinchliffe, *Science* **311**, 1430 (2006).
12. J. M. Berrisford, L. A. Sazanov, *J. Biol. Chem.* **284**, 29773 (2009).
13. M. L. Verkhovskaya, N. Belevich, L. Euro, M. Wikström, M. I. Verkhovsky, *Proc. Natl. Acad. Sci. U.S.A.* **105**, 3763 (2008).
14. D. Esterhazy, M. S. King, G. Yakovlev, J. Hirst, *Biochem.* **47**, 3964 (2008).
15. P. Hinchliffe, L. A. Sazanov, *Science* **309**, 771 (2005).
16. V. Zickermann *et al.*, *Biochim. Biophys. Acta* **1787**, 574 (2009).
17. C. Mathiesen, C. Hägerhäll, *Biochim. Biophys. Acta* **1556**, 121 (2002).
18. S. Kerscher, S. Dröse, K. Zwicker, V. Zickermann, U. Brandt, *Biochim. Biophys. Acta* **1555**, 83 (2002).
19. N. Morgner *et al.*, *Biochim. Biophys. Acta* **1777**, 1384 (2008).
20. A. Abdakhmanova *et al.*, *Biochim. Biophys. Acta* **1658**, 148 (2004).
21. Materials and methods are available as supporting material on Science Online.
22. E. Screpanti, C. Hunte, *J. Struct. Biol.* **159**, 261 (2007).
23. D. J. Morgan, L. A. Sazanov, *Biochim. Biophys. Acta* **1777**, 711 (2008).
24. E. A. Baranova, D. J. Morgan, L. A. Sazanov, *J. Struct. Biol.* **159**, 238 (2007).
25. G. Belevich, L. Euro, M. Wikström, M. Verkhovskaya, *Biochem.* **46**, 526 (2007).
26. K. Zwicker *et al.*, *J. Biol. Chem.* **281**, 23013 (2006).
27. M. A. Tocilescu, U. Fendel, K. Zwicker, S. Kerscher, U. Brandt, *J. Biol. Chem.* **282**, 29514 (2007).
28. U. Fendel, M. A. Tocilescu, S. Kerscher, U. Brandt, *Biochim. Biophys. Acta* **1777**, 660 (2008).
29. M. A. Tocilescu *et al.*, *Biochim. Biophys. Acta* **1797**, 625 (2010).
30. V. Zickermann *et al.*, *J. Biol. Chem.* **278**, 29072 (2003).
31. T. Clason, V. Zickermann, T. Ruiz, U. Brandt, M. Radermacher, *J. Struct. Biol.* **159**, 433 (2007).
32. T. Friedrich, *J. Bioenerg. Biomembr.* **33**, 169 (2001).
33. Excellent technical assistance by K. Siegmund and technical support by A. Duchene, F. Streb, and B. Wenzel are gratefully acknowledged. We thank S. Kerscher for his essential contributions regarding all aspects of *Y. lipolytica* genetics and B. Wrzesniewska for testing the impact of antibody fragments to improve crystallization. We are indebted to H. Michel for valuable advice during the initial stages of the project and for his continuous support. We thank the European Synchrotron Radiation Facility and the Swiss Light Source (SLS) for granting beam time, many scientists especially at SLS beamlines X06SA and X10SA for constantly supporting our work, and the staff of the Helmholtz Zentrum für Infektionsforschung (HZI)/Gesellschaft für Biotechnologische Forschung, Braunschweig for large-scale fermentation of *Y. lipolytica*. Funding by the Deutsche Forschungsgemeinschaft (SFB 472 Projects P2 and P17) is gratefully acknowledged. This study was supported by the Excellence Initiative of the German Federal and State Governments (EXC 115 and EXC 294). Electron density maps are available on request.

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1191046/DC1
Materials and Methods
Fig. S1
Tables S1 and S2
References

16 April 2010; accepted 10 June 2010

Published online 1 July 2010;

10.1126/science.1191046

Include this information when citing this paper.

An Electronic Bus Bar Lies in the Core of Cytochrome bc₁

Monika Świerczek,¹ Ewelina Cieluch,¹ Marcin Sarewicz,¹ Arkadiusz Borek,¹ Christopher C. Moser,² P. Leslie Dutton,² Artur Osyczka^{1*}

The ubiquinol–cytochrome c oxidoreductases, central to cellular respiration and photosynthesis, are homodimers. High symmetry has frustrated resolution of whether cross-dimer interactions are functionally important. This has resulted in a proliferation of contradictory models. Here, we duplicated and fused cytochrome b subunits, and then broke symmetry by introducing independent mutations into each monomer. Electrons moved freely within and between monomers, crossing an electron-transfer bridge between two hemes in the core of the dimer. This revealed an H-shaped electron-transfer system that distributes electrons between four quinone oxidation-reduction terminals at the corners of the dimer within the millisecond time scale of enzymatic turnover. Free and unregulated distribution of electrons acts like a molecular-scale bus bar, a design often exploited in electronics.

Figure 1 shows a bacterial ubiquinol–cytochrome c oxidoreductase (I), often called cytochrome bc₁, displaying homodi-

meric core subunit structure typical of respiratory and photosynthetic electron transfer systems (2, 3). It is well established that adjacent cofactors in each monomer serve to separate electronic charge across the membrane in the catalytically relevant microsecond-to-millisecond electron transfer process (4, 5). However, high structural, spectroscopic, and electrochemical symmetry between the monomers of the dimer has confounded efforts to determine whether a functional electron-transfer

connection exists between monomers. At the distances displayed in Fig. 1, calculations show that electron-tunneling times between cofactors in different monomers are much slower than the physiologically relevant time scale, except for tunneling between the two b_L hemes. Electron tunneling across the 13.9 Å separating these two hemes is calculated to be in the 0.025- to 0.25-ms range (5), slightly faster than the measured 0.5- to 5.0-ms physiological turnover time. However, electron-tunneling theory itself (6, 7) provides only an upper limit for the rate of electron transfer between redox cofactors. Many electron transfers in oxidoreductases are limited not by electron tunneling but by slower coupled events of chemistry, conformational change, or motion (8, 9). Indeed, many models have been proposed for ubiquinol–cytochrome c oxidoreductases that include just such regulation of electron transfer within or between monomers (10–15) or even strict electronic isolation of monomers (16). Given the inherent tunneling speed, a relatively small amount of coupling of this electron tunneling to chemical or conformational events could effectively regulate interaction between monomers or even isolate them (10–16).

To resolve the underlying dimer engineering, we broke the symmetry of the cytochrome bc₁ homodimer from *Rhodobacter capsulatus* shown

¹Department of Biophysics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland.

²The Johnson Research Foundation, Department of Biochemistry and Biophysics, University of Pennsylvania, PA 19104, USA.

*To whom correspondence should be addressed. E-mail: artur.osyczka@uj.edu.pl

Fig. 1. Cofactors and distances in homodimer of cytochrome *bc*₁ [Protein Data Bank ID: 1ZRT (1)]. Each monomer comprises cytochrome *b* (yellow), cytochrome *c*₁ (magenta), and FeS subunit (green). Functional distances (blue lines) and nonfunctional distances (red dashed lines) between cofactors (black) are in angstroms. Q_o site quinone is approximated from the crystallographic position of stigmatellin (1), and Q_i site quinone position is adopted from (28). FeS head domain movement (29) is indicated by the dashed arrow.

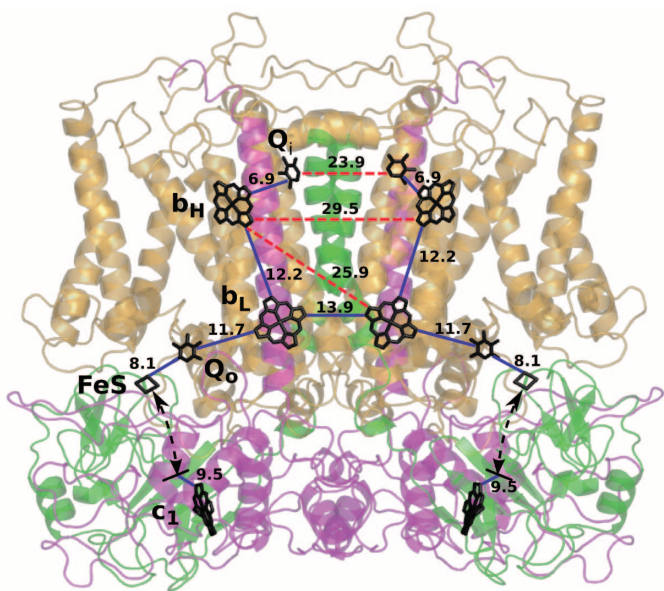
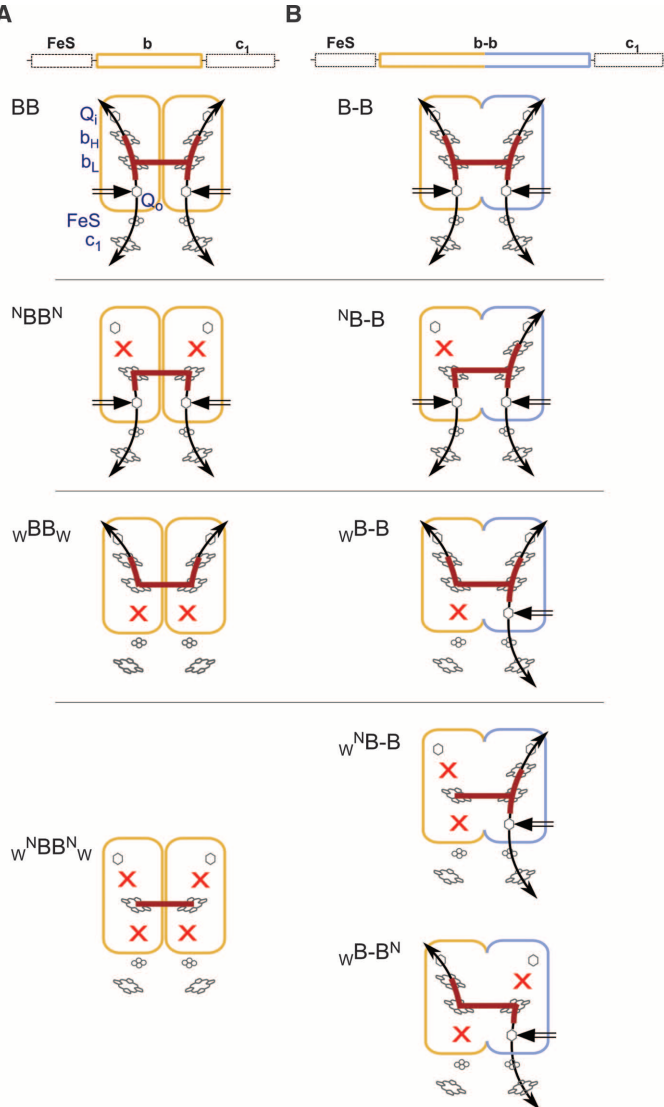


Fig. 2. Symmetric and asymmetric knockout patterns. Distribution of the knockouts (red crosses) constructed with unfused native operon coding (A) and fused gene coding (B). BB, native dimer: ^NBB^N, both upper branches removed; ^wBB^w, both lower branches removed; ^w^NBB^w, all four branches removed. B-B, fused protein: ^NB-B, one upper branch removed; ^wB-B, one lower branch removed; ^w^NB-B, two branches on the same side removed; ^wB-B^N, two branches across removed. N and W refer to H212N and G158W point mutations in cytochrome *b* (G, Gly; H, His; N, Asn; W, Trp). Black arrows, functional branches. Black double arrow, electron entry point at the Q_o site. Brown overlay: intraprotein electronic bus bar.



in Fig. 1. We genetically fused two identical monomeric cytochrome *b* subunits, analogous to the successful fusion of two membrane-anchored cytochromes *c* in *R. capsulatus* (17). The cytochrome *b* subunits accommodate the core cofactors in the electron-transfer chain and the putative bridge between monomers (Figs. 1 and 2). The N and C termini of the eight-transmembrane- α -helical chain of the monomeric cytochrome *b* protrude at the cytoplasmic side of the membrane. We joined these termini by extending the gene encoding cytochrome *b* with the linker peptide sequence followed by the second copy of the same gene containing Strep-tag at its C terminus (figs. S2 and S3) (18). The other two genes of the operon, encoding the subunits containing the FeS cluster and heme *c*₁, were left unchanged. Wild-type and fused cytochrome *bc*₁ are designated BB and B-B, respectively. Electrophoresis verified that the subunits of B-B had the correct molecular mass (fig. S4), and ultraviolet (UV)-visible and electron paramagnetic resonance spectroscopy (EPR) demonstrated normal cofactor assembly (Fig. 3 and figs. S5 and S6). Measurements of electron transfer in B-B (see below) indicated that the fused protein remained functional.

To uncover dimer-specific operation and test the putative H-shaped electron transfer system, we need only two asymmetrically positioned point mutations in B-B. We chose two sites that have been extensively characterized in BB. The mutation H212N (symmetrical ^NBB^N in Fig. 2A) prevents heme *b*_H assembly without affecting other cofactors in the cytochrome *bc*₁ structure (5). This heme *b*_H knockout markedly cuts short electron transfer in both upper H branches and, because upper and lower branches are energetically coupled, diminishes quinol oxidation levels at the Q_o site and linked electron transfer into the lower branch. The second site, G158W (symmetrical ^wBB^w in Fig. 2A), prevents substrate quinol binding at the Q_o site (Q_o site knockout), again without affecting the function of the other cofactors, and effectively inactivates dual electron transfer from quinol into both lower and upper branches (4). We achieved expression and assembly of mutants with asymmetrically placed copies of H212N or G158W in B-B, either separately or together in various combinations [table S1 and supporting online material (SOM) text]. Figure S4 confirms the proper size of the subunits of B-B in these asymmetric single- and double-mutant forms, and Fig. 3 and figs. S5 and S6 demonstrate that levels of expressed heme *b*_H (reported by UV-visible spectra) or occupied Q_o site (reported by the EPR spectrum of the FeS cluster) are precisely half that of the native cytochrome *bc*₁, as expected.

As depicted in Fig. 2B, permutations of these two strategically positioned mutations unambiguously expose all possible electron-transfer paths through the individual branches and bridge of this H-shaped electron transfer system. Figure 4 shows two types of kinetic assays, flash-induced on the left and steady-state on the right. After a flash of light activates the photosynthetic reaction

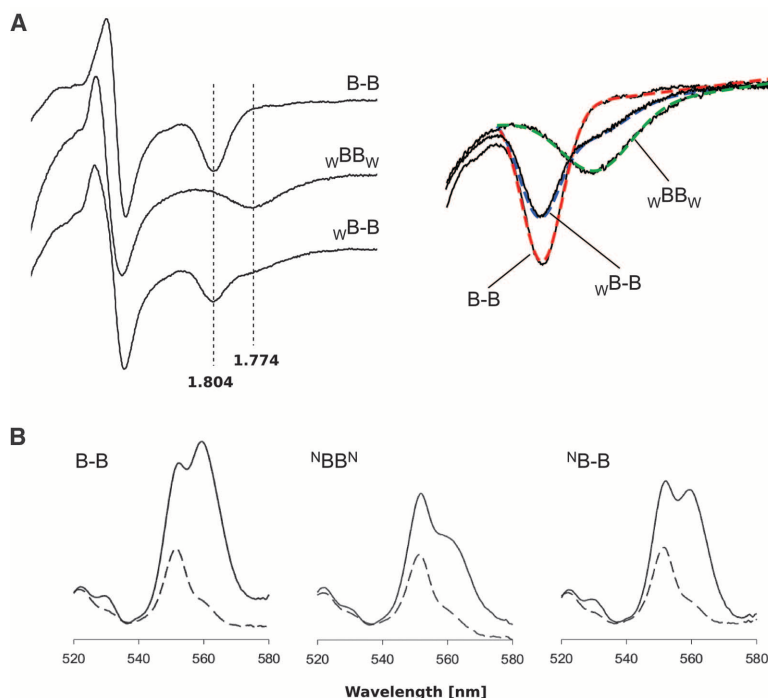


Fig. 3. Spectroscopic proof of structural asymmetry imposed by mutations in B-B. **(A)** X-band continuous-wave EPR spectra of the FeS cluster in membranes. Left: B-B with native g_x transition at 1.804 (intact Q_o site); wBB_W with g_x broadened and shifted to 1.774 (disabled Q_o site); $wB-B$ shows two distinct g_x transitions of 1.804 and 1.774. Right: g_x in $wB-B$ fitted with linear combinations of two Gaussian curves (blue) obtained from fitting of single Gaussian curve to the shape of g_x in B-B (red) and wBB_W (green) with equal contribution of each component. To produce such spectra, mutated and nonmutated Q_o sites in $wB-B$ must each communicate with one head domain of the FeS subunit, as expected for an assembly of one fusion protein per set of two FeS subunits. **(B)** Optical redox difference spectra of hemes in membranes: B-B with native-like spectrum with hemes C (peak at 550 nm) and B (peak at 560 nm) components; $^NBB^N$ with diminished amplitude at 560 nm reflecting absence of both hemes b_H in dimer; ^NB-B shows decreased peak at 560 nm with the amplitude in between that of the spectrum of B-B and $^NBB^N$, as expected for a loss of only one heme b_H in ^NB-B . Solid and dashed lines, dithionite minus ferricyanide and ascorbate minus ferricyanide spectra, respectively.

Table 1. Enzymatic activity supported by the complete H-shaped electron transfer system and its truncated derivatives.

Name*	Turnover rate [†] (1/s)	
	Without inhibitor	With antimycin [‡]
BB	56.4	3.2
$^NBB^N$	1.6	1.7
wBB_W	0.1	0.1
B-B	60.0	3.4
^NB-B	48.7	3.2
$wB-B$ (B- B_W)	51.9 (53.6)	2.0 (2.1)
w^NB-B (B- B^N_W)	36.4 (46.0)	1.9 (2.0)
$wB-B^N$	26.1	1.3

*Letter code corresponds to schemes of Fig. 2. Obtainable second versions of some forms (table S1) and their activities are shown in parentheses. [†]Measured for cytochrome bc_1 in membranes. [‡]Myxothiazol or stigmatellin in place of antimycin abolished activity to almost zero in all forms.

center to oxidize cytochrome c, the re-reduction of cytochrome c provides a sensitive indicator of electron-transfer activity and quinol oxidation catalysis in cytochrome bc_1 . Cytochrome c oxidation-reduction in B-B (Fig. 4A, top trace) is similar to that of wild-type BB (not shown). Flash-activated microsecond oxidation of cytochrome c is shown as a prompt downward change. In the ensuing milliseconds, the upward trending cytochrome c trace

shows re-reduction by electrons coming through the lower branches of the H from oxidations of quinol in the Q_o site (Fig. 4A, top trace, black).

The critical involvement of upper and lower branches in quinol oxidation is demonstrated by inhibition by antimycin. It inactivates both Q_i sites and prevents movement of electrons through and out of the upper branches, which in turn restricts movement of electrons through and out of the lower

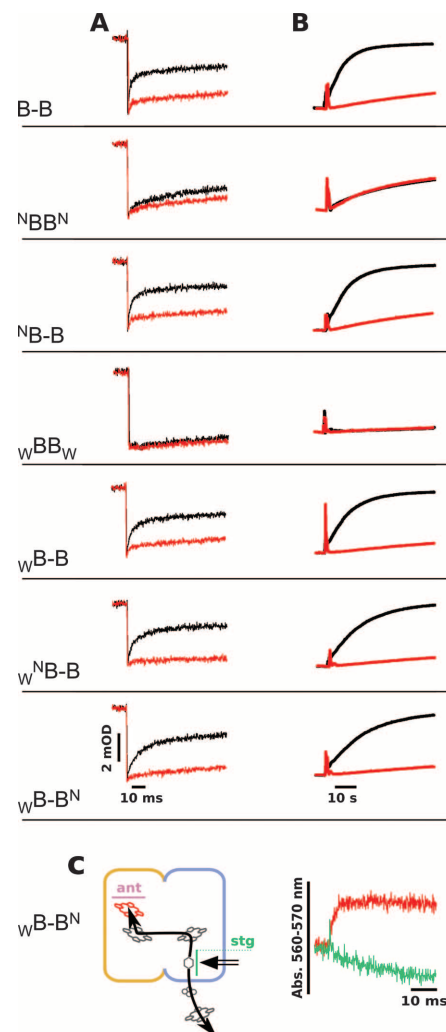


Fig. 4. Testing functional branch connection in the H-shaped electron transfer system. **(A)** Light-induced oxidation and re-reduction of cytochrome c at 550 minus 540 nm in membranes containing complete knockout variations described in Fig. 2. Black, uninhibited; red, inhibited with antimycin. $B-B_W$ and $B-B^N_W$ displayed kinetics similar to that of $wB-B$ and w^NB-B , respectively (not shown). **(B)** Corresponding steady-state enzymatic reduction of cytochrome c at 550 nm. Rates are listed in Table 1. **(C)** Light-induced heme b_H kinetics in $wB-B^N$ in the presence of antimycin abolishing Q_i action (red) or stigmatellin abolishing Q_o action (green). In $wB-B^N$ blocked with antimycin (ant), the only route to reduce heme b_H (red) must involve the heme b_L to b_L electron transfer. stg, stigmatellin.

branch to cytochrome c; thus cytochrome c reduction is greatly impeded (Fig. 4A, top trace, red). Similarly, the double, symmetric heme b_H knockout ($^NBB^N$) trims the upper branch at the point before the Q_i site and impedes cytochrome c reduction, even without antimycin (Fig. 4A, second trace). However, unlike the symmetric $^NBB^N$, the single asymmetric heme b_H knockout (^NB-B) that inactivates one of the two upper branches has cytochrome c re-reduction kinetics very similar to that of the knockout-free B-B (Fig. 4A, third trace). Parallel results are

found for the Q_o site mutants. Cytochrome *c* re-reduction in the double, symmetrical Q_o site knockout (wBB_w) is impeded (Fig. 4A, fourth trace), whereas the inactivation of only one of the two lower branches (either $wB-B$ or $B-B_w$) reveals cytochrome *c* re-reduction kinetics only slightly slower than in knockout-free $B-B$ (Fig. 4A, fifth trace). Moreover, knocking out both upper and lower branches of electron transfer in the same monomer (w^NB-B or $B-B^N_w$) causes a similar minor slowing of cytochrome *c* re-reduction (Fig. 4A, second from bottom). These results demonstrate that the activity of one intact monomer is independent of the functional status of the other monomer.

The mutant combination $wB-B^N$ (Fig. 2B and Fig. 4A, bottom) allows investigation of cross monomer electron transfer. The result is unambiguous. After light-flash-induced oxidation of cytochrome *c*, re-reduction follows the same general pattern observed with the unmutated $B-B$ and mutants with singly or doubly knocked out cofactors in the same chain of one monomer. Thus, flash-activated electron transfer occurs between the monomers on the millisecond time scale.

Figure 4B and Table 1 repeat these analyses with a dark, steady-state activity assay that is standard for cytochrome bc_1 in respiratory systems. These analyses confirm the results from flash activation in showing that for ^NB-B , $wB-B$ or $B-B_w$, w^NB-B or $B-B^N_w$, and $wB-B^N$ the observed steady-state rates are never less than half that of $B-B$.

Figure 4C reveals approximately millisecond electron transfer across the bridge in $wB-B^N$ more directly by following heme b_H reduction. This roughly matches enzymatic turnover and demonstrates that intermonomer electron transfer is a physiologically relevant event. This time is also within the uncertainty of the calculated pure tunneling time, showing that any chemical or conformational events coupled to electron tunneling between hemes b_L must be minor, and is inconsistent with the view that electron transfer between the hemes b_L does not occur (16). Moreover, because inactivation of either monomer or any upper or lower monomer branch has no notable effect on the milliseconds or seconds time scale electron transfer throughout the dimer, there is serious doubt that intermonomer or interbranch conformational interactions play an important role in regulating energy coupling or function of cytochrome bc_1 (10–15).

In the absence of intricate regulation, the natural engineering of electron transfer connections in dimeric cytochrome bc_1 appears relatively simple and robust. Dimerization of proteins is common and proceeds for any number of different reasons. But merely by permitting two of the core redox cofactors on either side of a dimer interface to approach to within a 14 Å electron-tunneling distance, independent elementary redox chains are converted to an H-shaped electron transfer system that enables any connection between terminals on opposite sides of the membrane to be enzymatically competent. This simple electronic distribution to the four terminals of the dimer can be likened to a molecular version of a conducting bus bar familiar

in electronics (fig. S1). The uniting action of the bus bar offers several advantages for respiration and photosynthesis, especially under stress. Multiple unpaired electrons produced at the Q_o site (19, 20) and implicated in the production of reactive oxygen species (ROS) (21–24) can be collected and neutralized (5, 25). The bus bar also builds in redundancy to allow physiological function of the protein even after operational damage of one part, perhaps from ROS. Finally, this design increases the effective diffusion target size for substrates ubiquinone and cytochrome *c* (26) that may be critical in the overcrowded bioenergetic membrane (27).

References and Notes

1. E. A. Berry *et al.*, *Photosynth. Res.* **81**, 251 (2004).
2. E. A. Berry, M. Guergova-Kuras, L. S. Huang, A. R. Crofts, *Annu. Rev. Biochem.* **69**, 1005 (2000).
3. W. A. Cramer, H. Zhang, J. Yan, G. Kurisu, J. L. Smith, *Annu. Rev. Biochem.* **75**, 769 (2006).
4. H. Ding *et al.*, *Biochemistry* **34**, 15979 (1995).
5. A. Osyczka, C. C. Moser, F. Daldal, P. L. Dutton, *Nature* **427**, 607 (2004).
6. C. C. Moser, J. M. Keske, K. Warncke, R. S. Farid, P. L. Dutton, *Nature* **355**, 796 (1992).
7. C. C. Page, C. C. Moser, X. Chen, P. L. Dutton, *Nature* **402**, 47 (1999).
8. C. C. Moser, S. E. Chobot, C. C. Page, P. L. Dutton, *Biochim. Biophys. Acta* **1777**, 1032 (2008).
9. C. C. Moser, C. C. Page, P. L. Dutton, *Phil. Trans. R. Soc. B* **361**, 1295 (2006).
10. A. A. Gupta, B. A. Feniouk, W. Junge, A. Y. Mulikdjanian, *FEBS Lett.* **431**, 291 (1998).
11. A. Y. Mulikdjanian, *Photochem. Photobiol. Sci.* **6**, 19 (2007).
12. R. Covian, B. L. Trumpower, *Biochim. Biophys. Acta* **1777**, 1079 (2008).
13. C. A. Yu *et al.*, *Biochim. Biophys. Acta* **1777**, 1038 (2008).
14. J. W. Cooley, D.-W. Lee, F. Daldal, *Biochemistry* **48**, 1888 (2009).

15. M. Castellani *et al.*, *J. Biol. Chem.* **285**, 502 (2010).
16. A. R. Crofts *et al.*, *Biochim. Biophys. Acta* **1777**, 1001 (2008).
17. D.-W. Lee, Y. Öztürk, A. Osyczka, J. W. Cooley, F. Daldal, *J. Biol. Chem.* **283**, 13973 (2008).
18. Materials and methods are available as supporting material on Science Online.
19. J. L. Cape, M. K. Bowman, D. M. Kramer, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 7887 (2007).
20. H. Zhang, A. Osyczka, P. L. Dutton, C. C. Moser, *Biochim. Biophys. Acta* **1767**, 883 (2007).
21. M. Ksenzenko, A. A. Konstantinov, G. B. Khomutov, A. N. Tikhonov, E. K. Ruuge, *FEBS Lett.* **155**, 19 (1983).
22. F. Muller, A. R. Crofts, D. M. Kramer, *Biochemistry* **41**, 7866 (2002).
23. S. Dröse, U. Brandt, *J. Biol. Chem.* **283**, 21649 (2008).
24. A. Borek, M. Sarewicz, A. Osyczka, *Biochemistry* **47**, 12365 (2008).
25. V. P. Shinkarev, C. A. Wraight, *FEBS Lett.* **581**, 1535 (2007).
26. M. Sarewicz, A. Borek, F. Daldal, W. Francisz, A. Osyczka, *J. Biol. Chem.* **283**, 24826 (2008).
27. R. J. Ellis, *Trends Biochem. Sci.* **26**, 597 (2001).
28. C. Hunte, J. Koepke, C. Lange, T. Rossmanith, H. Michel, *Structure* **8**, 669 (2000).
29. E. Darrouzet, C. C. Moser, P. L. Dutton, F. Daldal, *Trends Biochem. Sci.* **26**, 445 (2001).
30. We are grateful to F. Daldal (University of Pennsylvania, Philadelphia, USA) for the genetic system for cytochrome bc_1 in *R. capsulatus*. This work was supported by The Wellcome Trust International Senior Research Fellowship (A.O.) and by the United States National Institutes of Health (P.L.D.).

Supporting Online Material

www.sciencemag.org/cgi/content/full/329/5990/451/DC1

Materials and Methods

SOM Text

Figs. S1 to S6

Table S1

References

14 April 2010; accepted 9 June 2010

10.1126/science.1190899

Sfrp5 Is an Anti-Inflammatory Adipokine That Modulates Metabolic Dysfunction in Obesity

Noriyuki Ouchi,^{1*} Akiko Higuchi,¹ Koji Ohashi,¹ Yuichi Oshima,¹ Noyan Gokce,² Rei Shibata,³ Yuichi Akasaki,¹ Akihiko Shimono,⁴ Kenneth Walsh^{1*}

Adipose tissue secretes proteins referred to as adipokines, many of which promote inflammation and disrupt glucose homeostasis. Here we show that secreted frizzled-related protein 5 (Sfrp5), a protein previously linked to the Wnt signaling pathway, is an anti-inflammatory adipokine whose expression is perturbed in models of obesity and type 2 diabetes. Sfrp5-deficient mice fed a high-calorie diet developed severe glucose intolerance and hepatic steatosis, and their adipose tissue showed an accumulation of activated macrophages that was associated with activation of the c-Jun N-terminal kinase signaling pathway. Adenovirus-mediated delivery of Sfrp5 to mouse models of obesity ameliorated glucose intolerance and hepatic steatosis. Thus, in the setting of obesity, Sfrp5 secretion by adipocytes exerts salutary effects on metabolic dysfunction by controlling inflammatory cells within adipose tissue.

Obesity is a predisposing factor for metabolic disorders, such as type 2 diabetes, which are often associated with a low-grade inflammatory state in adipose tissue. Adipose tissue secretes a variety of cytokines, referred to as adipokines (1–3). Most adipokines—such as tumor necrosis factor α (TNF α), interleukin-6 (IL-6), and leptin—are proinflammatory. One

prominent exception is adiponectin (APN), an anti-inflammatory adipokine that promotes insulin sensitization and protects cardiovascular tissue from ischemic injury (2, 4).

Because adipokine dysregulation can contribute to the pathogenesis of obesity-linked disorders, we sought to identify new adipokines by comparing the gene expression profile of adipose tissue

from lean mice with that from obese mice fed a high-fat, high-sucrose (HF-HS) diet (5, 6). Of numerous candidates, we found that secreted frizzled-related protein (Sfrp) 5 was expressed at substantially higher levels in white adipose tissue than in other tissues at both the transcript and protein levels and that the Sfrp5 transcript was confined to adipocytes rather than stromal vascular cells (fig. S1). Sfrp proteins sequester Wnt proteins in the extracellular space and prevent Wnt binding to their receptors (7, 8). Canonical Wnt signaling negatively regulates adipogenesis (9), but little is known about the role of Sfrp5 in the control of noncanonical Wnt signaling in adipose tissue or in the regulation of systemic metabolism.

To investigate Sfrp5 regulation during metabolic dysfunction, we examined its expression in rodent models of obesity (6). Sfrp5 expression was reduced in obese leptin-deficient (ob/ob) mice (Fig. 1A) and Zucker diabetic fatty rats (fig. S2). Sfrp5 expression was also reduced in wild-type (WT) mice fed a HF-HS diet for 24 weeks (Fig. 1B), but Sfrp5 expression was transiently up-regulated after 12 weeks of the diet, when metabolic dysfunction and adipose tissue expression of markers of inflammation, macrophage infiltration, and oxidative and endoplasmic reticulum stresses are less severe (fig. S3). Sfrp5 has been shown to bind and antagonize both Wnt5a and Wnt11 (10). Wnt5a protein expression, but not Wnt11, could be detected in adipose tissues (Fig. 1 and figs. S2 and S3). In all models examined, obesity led to higher levels of Wnt5a expression and an increase in the ratio of Wnt5a to Sfrp5.

To determine whether Sfrp5 regulation is relevant to human obesity, we analyzed visceral fat biopsy specimens of obese individuals. Individuals presenting with macrophage crown-like structures (CLSs), an indicator of adipose tissue inflammation (11, 12), also displayed a decrease in Sfrp5 transcript expression compared with obese individuals that were negative for CLS (fig. S4). CLS-positive individuals also displayed higher levels of adipose TNF α transcript expression and an increase in the homeostasis assessment model for insulin resistance (HOMA-IR) index.

To investigate whether Sfrp5 regulates metabolism, we studied Sfrp5-deficient (*Sfrp5*^{-/-}) mice fed normal or HF-HS diets. No significant differences in body weight (BW) (WT mice: 33.1 $\pm$ 0.8 g and *Sfrp5*^{-/-} mice: 34.1 $\pm$ 1.1 g), glucose disposal, or insulin sensitivity could be detected between *Sfrp5*^{-/-} and WT mice when mice were given standard feed (Fig. 2A). However, feeding the HF-

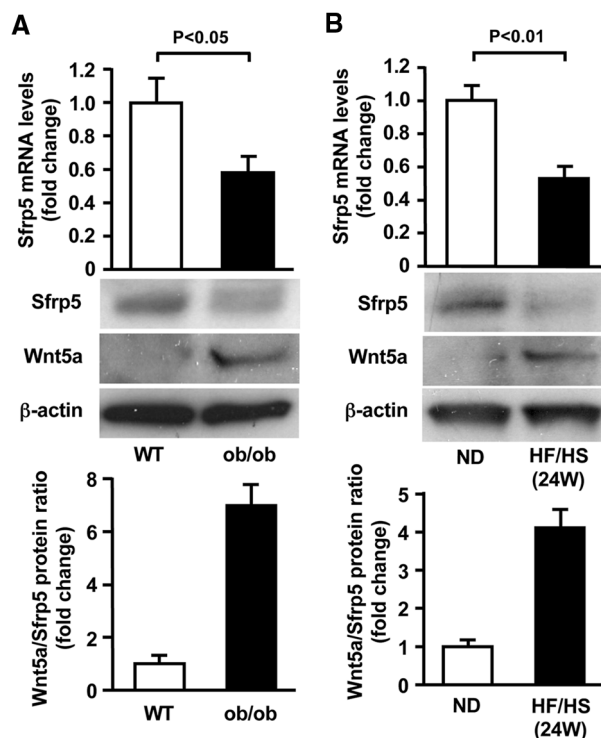
HS diet to *Sfrp5*^{-/-} mice for 12 weeks led to significant impairments in glucose clearance and insulin sensitivity compared with results in WT mice (Fig. 2A). Although *Sfrp5*^{-/-} mice fed a HF-HS diet showed a small, but significant, increase in BW compared with WT mice, both strains had similar daily food intake during the experimental period of HF-HS diet feeding (fig. S5). Fasting glucose and insulin levels were elevated, and a greater degree of hepatic steatosis, with a higher triglyceride content and heavier liver weight, were observed in *Sfrp5*^{-/-} mice compared with WT mice fed the HF-HS diet (Fig. 2B and fig. S5). We also performed histological analyses on epididymal adipose tissues. *Sfrp5*^{-/-} mice fed a HF-HS diet had larger adipocytes than WT mice fed the same diet (Fig. 2C). Thus, Sfrp5-deficiency produces a phenotype of metabolic dysfunction only in mice that are under obesity-induced metabolic stress and not in mice under normal nutritional conditions.

The development of insulin resistance is linked to a macrophage-mediated inflammation of adipose tissues (13–15). Thus, to determine whether *Sfrp5*^{-/-} mice show signs of an increased inflammatory response, the macrophage content of adipose tissue from *Sfrp5*^{-/-} and WT mice was measured. Cells positive for F4/80, a macrophage marker, were more abundant in adipose tissue of *Sfrp5*^{-/-} mice than that of WT mice when both strains were fed a HF-HS diet (Fig. 2D). Consistent with this finding, transcript levels of F4/80 and CD68, another macrophage marker, were significantly elevated in epididymal adipose tissue of *Sfrp5*^{-/-} mice compared with WT mice fed a HF-HS diet, but not when given normal feed (fig. S6). We also examined the expression levels of proinflammatory mediators in the stromal vascular

fractions isolated from epididymal adipose tissue. When mice were fed a HF-HS diet, significant increases in levels of TNF α , IL-6, and monocyte chemoattractant protein-1 (MCP-1) transcripts occurred in the stromal vascular fraction from fat tissue of *Sfrp5*^{-/-} mice compared with WT mice (fig. S6). Transcript levels of TNF α , IL-6, and MCP-1 did not differ between *Sfrp5*^{-/-} and WT mice when given normal feed.

To analyze the downstream signaling pathways affected by Sfrp5 deficiency, we assessed whether the canonical or noncanonical Wnt signaling pathways were activated in epididymal adipose tissues of *Sfrp5*^{-/-} and WT mice fed a HF-HS diet. No differences were detected in transcript expression of cyclin D1 or WISP-2, indicators of canonical Wnt signaling, between *Sfrp5*^{-/-} and WT mice (fig. S6). In contrast, phosphorylation of c-Jun N-terminal kinase (JNK), a downstream target of the noncanonical Wnt signaling (16, 17), was elevated 2.0 $\pm$ 0.1 (P < 0.05) in white adipose tissue in *Sfrp5*^{-/-} mice. Also, the phosphorylation of c-Jun, a downstream substrate of JNK, was elevated in *Sfrp5*^{-/-} mice by a factor of 2.3 $\pm$ 0.2 (P < 0.05) compared with WT mice (Fig. 3A). Activation of JNK1 promotes insulin resistance through serine phosphorylation of insulin receptor substrate-1 (IRS-1) (18). We found that IRS-1 phosphorylation at residue Ser³⁰⁷ was increased in fat tissue of *Sfrp5*^{-/-} mice by a factor of 2.2 $\pm$ 0.3 (P < 0.05) compared with WT mice (Fig. 3A). We also assessed insulin signaling in adipose tissue by measuring the activating phosphorylation of Akt at Ser⁴⁷³ after systemic insulin administration. In WT mice fed a HF-HS diet, insulin stimulated the phosphorylation of Akt in fat pads, but this in-

Fig. 1. Expression of Sfrp5 in white adipose tissue in lean and obese mice. **(A)** Expression of Sfrp5 and Wnt5a in epididymal fat tissue in WT and ob/ob mice at the age of 20 weeks. **(B)** Expression of Sfrp5 and Wnt5a in epididymal adipose tissue of WT mice fed normal diet (ND) or HF-HS diet for 24 weeks. Sfrp5 transcript levels were measured by quantitative real-time polymerase chain reaction analysis and expressed relative to a reference gene, 36B4 (n = 6 or 7 mice). Expression of Sfrp5 and Wnt5a protein was determined by immunoblot analysis.



¹Molecular Cardiology and Whitaker Cardiovascular Institute, Boston University School of Medicine, 715 Albany Street, W611, Boston, MA 02118, USA. ²Evans Department of Medicine and Whitaker Cardiovascular Institute, Boston University School of Medicine, 715 Albany Street, E703E, Boston, MA 02118, USA. ³Department of Cardiology, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya 466-8550, Japan. ⁴Cancer Science Institute of Singapore, National University of Singapore, Centre of Life Sciences, 28 Medical Drive, no. 02-07, Singapore 117456.

*To whom correspondence should be addressed: E-mail: kxwalsh@bu.edu (K.W.); nouchi@bu.edu (N.O.)

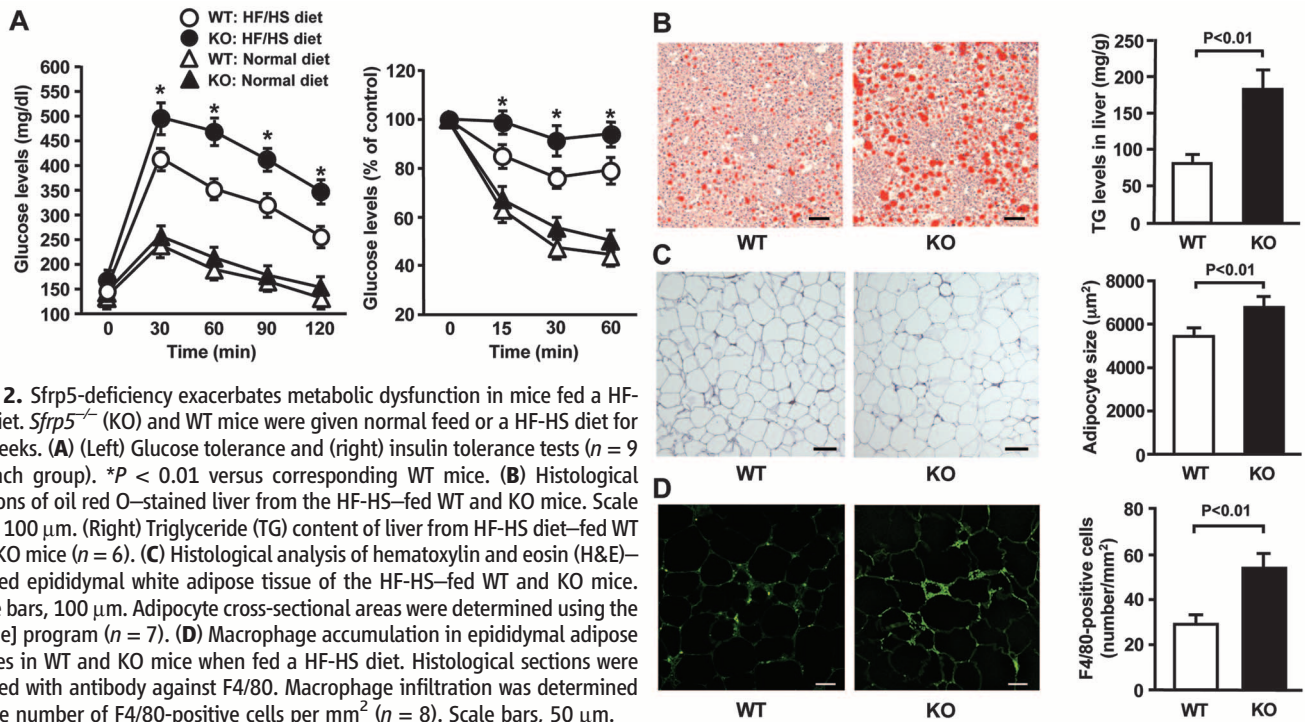
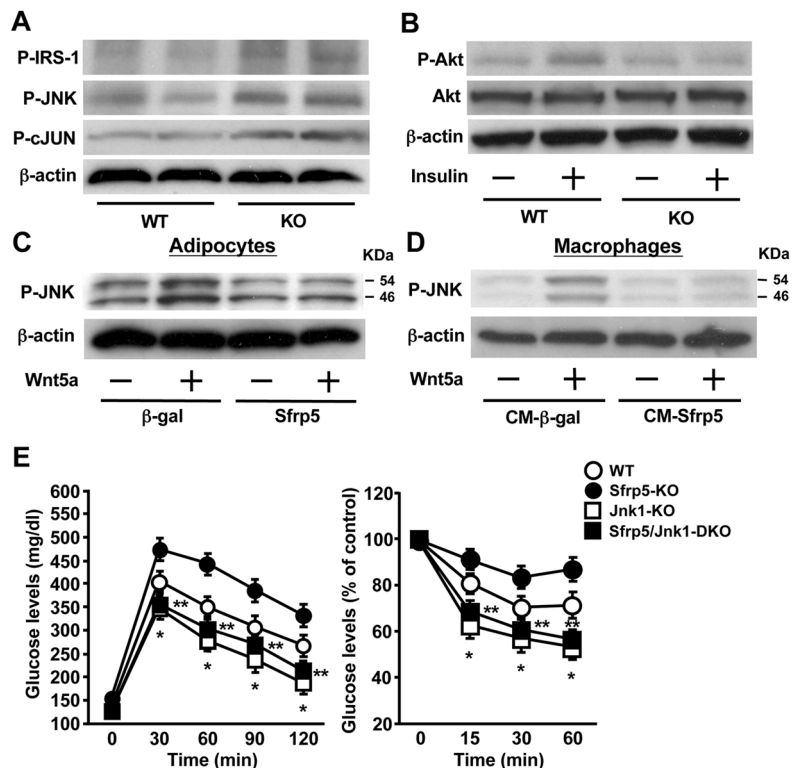


Fig. 2. *Sfrp5*-deficiency exacerbates metabolic dysfunction in mice fed a HF-HS diet. *Sfrp5*^{-/-} (KO) and WT mice were given normal feed or a HF-HS diet for 12 weeks. (A) (Left) Glucose tolerance and (right) insulin tolerance tests (*n* = 9 in each group). **P* < 0.01 versus corresponding WT mice. (B) Histological sections of oil red O-stained liver from the HF-HS-fed WT and KO mice. Scale bars, 100 μm. (Right) Triglyceride (TG) content of liver from HF-HS diet-fed WT and KO mice (*n* = 6). (C) Histological analysis of hematoxylin and eosin (H&E)-stained epididymal white adipose tissue of the HF-HS-fed WT and KO mice. Scale bars, 100 μm. Adipocyte cross-sectional areas were determined using the ImageJ program (*n* = 7). (D) Macrophage accumulation in epididymal adipose tissues in WT and KO mice when fed a HF-HS diet. Histological sections were stained with antibody against F4/80. Macrophage infiltration was determined as the number of F4/80-positive cells per mm² (*n* = 8). Scale bars, 50 μm.

Fig. 3. JNK1 activation contributes to diet-induced metabolic dysfunction in *Sfrp5*-deficient mice and Wnt5a-mediated cell activation in vitro. *Sfrp5*^{-/-} (KO) and WT mice were maintained on a HF-HS diet for 12 weeks. (A) Phosphorylation of JNK (Thr¹⁸³/Tyr¹⁸⁵), cJUN (Ser⁶³), and IRS-1 (Ser³⁰⁷) in fat tissue of WT and KO mice as determined by immunoblot analysis. (B) Akt phosphorylation in adipose tissues of WT and KO mice after insulin administration. (C) Effect of *Sfrp5* on Wnt5a-stimulated JNK phosphorylation in adipocytes. 3T3-L1 adipocytes were transduced with Ad-β-gal or Ad-*Sfrp5* in the presence of AdCMV-tTA, which uses a cytomegalovirus promoter sequence to drive a tetracycline transactivator and activate β-galactosidase or *Sfrp5*, followed by treatment with Wnt5a or vehicle for 30 min. (D) Effect of the conditioned medium from *Sfrp5*-transfected adipocytes on Wnt5a-induced JNK activation in macrophages. Peritoneal macrophages were stimulated with Wnt5a or vehicle for 30 min in the presence of the conditioned media from 3T3-L1 adipocyte transduced with Ad-β-gal or Ad-*Sfrp5* along with AdCMV-tTA. (E) Contribution of JNK1 to severe insulin resistance caused by *Sfrp5* deficiency. WT, *Sfrp5*^{-/-} (*Sfrp5*-KO), *Jnk1*^{-/-} (*Jnk1*-KO), and *Sfrp5*^{-/-} *Jnk1*^{-/-} (*Sfrp5*/*Jnk1*-DKO) mice were maintained on a HF-HS diet for 12 weeks. (Left) Glucose tolerance and (right) insulin tolerance tests were performed (*n* = 6 or 7 in each group). **P* < 0.01 versus WT mice. ***P* < 0.01 versus *Sfrp5*-KO mice.

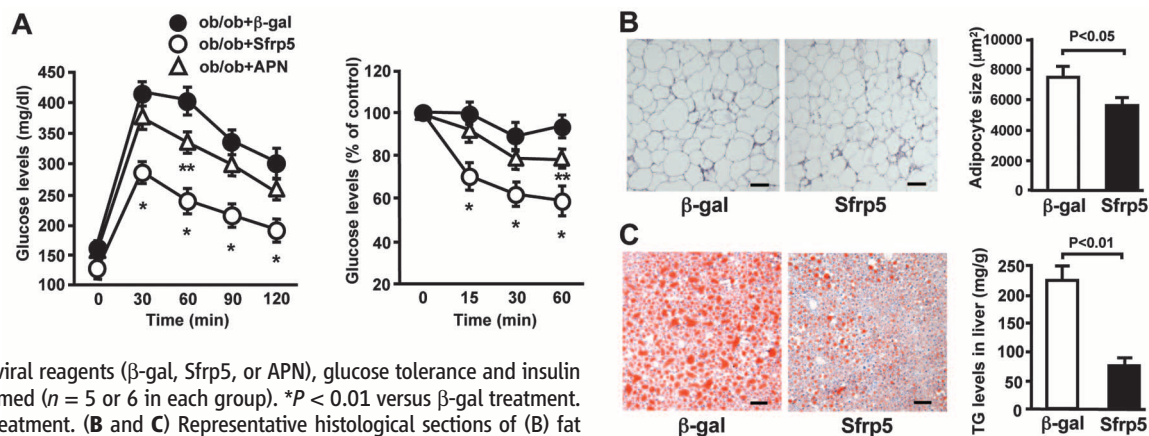


duction of phosphorylation was diminished in the HF-HS diet-fed *Sfrp5*^{-/-} mice (Fig. 3B). Because activation of JNK causes obesity-induced insulin resistance (18), we hypothesized that the enhanced metabolic dysfunction observed in HF-HS diet-fed *Sfrp5*^{-/-} mice could be attributed to the noncanonical activation of JNK in fat tissues.

To determine the effect of *Sfrp5* and Wnt5a on JNK activation at the cellular level, we transduced 3T3-L1 adipocytes with adenoviral vectors encoding *Sfrp5* (Ad-*Sfrp5*) or β-galactosidase (Ad-β-gal) as controls, then incubated the cells with Wnt5a protein. Compared with control vector, Ad-*Sfrp5* increased *Sfrp5* protein levels in both

cell lysates and media (fig. S7) and cancelled the stimulatory effects of Wnt5a on the phosphorylation of JNK in adipocytes (Fig. 3C). Consistent with findings from mice, neither *Sfrp5* nor Wnt5a had an effect on TOPflash reporter activity, which transcriptionally responds to canonical Wnt signaling (fig. S7).

Fig. 4. Systemic delivery of Sfrp5 is protective against metabolic dysfunction in obese mice. (A) (Left) Glucose tolerance and (right) insulin tolerance assays. Ad- β -gal and Ad-Sfrp5 along with AdCMV-tTA, or Ad-APN were intravenously administered to ob/ob mice at the age of 20 weeks. Two weeks after supplementation of adenoviral reagents (β -gal, Sfrp5, or APN), glucose tolerance and insulin tolerance tests were performed ($n = 5$ or 6 in each group). * $P < 0.01$ versus β -gal treatment. ** $P < 0.05$ versus β -gal treatment. (B and C) Representative histological sections of (B) fat pads stained with H&E and (C) liver stained with oil red O in β -gal- or Sfrp5-treated ob/ob mice. Scale bars, 100 μ m. (B) (Right) Quantification of adipocyte size ($n = 6$). (C) (Right) Triglyceride (TG) content of liver ($n = 6$).



To assess the effect of Sfrp5 on JNK activation and inflammatory responses in macrophages *in vitro*, we stimulated murine macrophages with Wnt5a protein in the presence of conditioned media from 3T3-L1 adipocytes transduced with Ad-Sfrp5 or Ad- β -gal. Wnt5a-stimulated JNK phosphorylation in macrophages was blocked by the conditioned medium from adipocytes transduced with Ad-Sfrp5, but not Ad- β -gal (Fig. 3D). Treatment with Wnt5a also increased TNF α and IL-6 transcript expression by macrophages, and this was also blocked by conditioned medium from Ad-Sfrp5-transduced adipocytes (fig. S8). To explore whether JNK signaling contributes to Wnt5a-stimulated induction of TNF α and IL-6, we pretreated macrophages with the JNK inhibitor SP600125 and incubated the cells with Wnt5a. Pretreatment with SP600125 diminished Wnt5a-stimulated expression of TNF α and IL-6 (fig. S8), which indicated that Sfrp5 blocks macrophage activation through inhibition of Wnt5a-JNK signaling. Similarly, the stimulatory effect of Wnt5a on IL-6 expression in adipocytes was blocked by transduction with Ad-Sfrp5 or pretreatment with SP600125 (fig. S7).

To investigate the role of JNK1 activation in the dysfunctional metabolic phenotype of the Sfrp5-deficient mice, we generated mice lacking both *Sfrp5* and *Jnk1*. Consistent with a previous report (18), *Jnk1*^{-/-} mice exhibited improvements in insulin and glucose sensitivity, as well as reduced BW, compared with WT mice when fed the HF-HS diet (Fig. 3E and fig. S9). Whereas *Sfrp5*^{-/-} mice showed profound insulin resistance and glucose intolerance, *Sfrp5*^{-/-}*Jnk1*^{-/-} double-knockout (DKO) mice showed glucose disposal responses that were comparable with those of *Jnk1*^{-/-} mice (Fig. 3E). Furthermore, although BW and transcript levels of TNF α , IL-6, and MCP-1 in fat tissue were elevated in *Sfrp5*^{-/-} mice compared with WT mice, BW and proinflammatory mediator expression levels did not differ for *Jnk1*^{-/-} and *Sfrp5*^{-/-}*Jnk1*^{-/-} mice (fig. S9). Thus, the impaired insulin sensitivity and enhanced adipose tissue inflammation in *Sfrp5*^{-/-} mice can be attributed to enhanced activation of JNK1.

To assess whether Sfrp5 can prevent the development of insulin resistance *in vivo*, we intravenously administered Ad-Sfrp5 or Ad- β -gal to WT and *Sfrp5*^{-/-} mice that were fed a HF-HS diet. Both WT and *Sfrp5*^{-/-} mice treated with Ad-Sfrp5 showed significant improvements in glucose clearance compared with mice treated with the control vector (fig. S10). To investigate the effect of Sfrp5 treatment on glucose metabolism in another model of metabolic dysfunction, we delivered Ad-Sfrp5 or Ad- β -gal into ob/ob mice. Parallel experiments examined the consequences of intravenous injection of an adenoviral vector expressing APN (Ad-APN), because the chronic overexpression of this adipokine has been shown to reverse the metabolic consequences of leptin deficiency (19). Two weeks after treatment with Ad-Sfrp5, glucose and insulin sensitivity were significantly improved (Fig. 4A). The administration of Ad-APN to ob/ob mice led to a threefold increase in plasma APN levels, but this was relatively ineffective in improving glucose clearance in this model. The administration of Ad-Sfrp5 also led to significant reductions in transcript levels of TNF α , IL-6, MCP-1, F4/80, and CD68 in adipose tissue, and this was accompanied by a reduction in the activating phosphorylation of JNK (fig. S11). Treatment with Ad-Sfrp5 also led to the atrophy of enlarged white adipocytes in ob/ob mice (Fig. 4B) with a reduction of fat weight (Ad- β -gal: 2.51 $\pm$ 0.19 g and Ad-Sfrp5: 2.01 $\pm$ 0.11 g, $P < 0.05$) and a marked attenuation of lipid accumulation in the liver (Fig. 4C). Taken together, these data indicate that acute Sfrp5 administration can reverse hyperglycemia and hepatic steatosis in multiple mouse models of metabolic dysfunction.

In summary, we show that Sfrp5 is secreted by adipocytes and that it controls the microenvironment of white adipose tissue under conditions of metabolic stress. Whereas *Sfrp5*^{-/-} mice do not express a detectable phenotype when given normal feed, these animals displayed aggravated fat pad inflammation and systemic metabolic dysfunction when fed a high-calorie diet. Conversely, the acute administration of Sfrp5 to models of obese and diabetic mice improved metabolic function

and reduced adipose tissue inflammation. We propose that Sfrp5 neutralizes noncanonical JNK activation by Wnt5a in macrophages and adipocytes via paracrine and autocrine mechanisms, respectively. The JNK signaling pathway in adipocytes and macrophages has emerged as an important mediator of adipose tissue inflammation that affects systemic metabolism (18, 20–22). Thus, the Sfrp5-JNK1 regulatory axis in fat represents a potential target for the control of obesity-linked abnormalities in glucose homeostasis.

References and Notes

- B. M. Spiegelman, J. S. Flier, *Cell* **87**, 377 (1996).
- N. Ouchi, S. Kihara, T. Funahashi, Y. Matsuzawa, K. Walsh, *Curr. Opin. Lipidol.* **14**, 561 (2003).
- P. E. Scherer, *Diabetes* **55**, 1537 (2006).
- R. Shibata *et al.*, *Nat. Med.* **11**, 1096 (2005).
- Y. Izumiya *et al.*, *Cell Metab.* **7**, 159 (2008).
- Materials and methods are available as supporting material on Science Online.
- P. Bovolenta, P. Esteve, J. M. Ruiz, E. Cisneros, J. Lopez-Rios, *J. Cell Sci.* **121**, 737 (2008).
- Y. Kawano, R. Kypta, *J. Cell Sci.* **116**, 2627 (2003).
- S. E. Ross *et al.*, *Science* **289**, 950 (2000).
- Y. Li *et al.*, *Genes Dev.* **22**, 3050 (2008).
- C. M. Apovian *et al.*, *Arterioscler. Thromb. Vasc. Biol.* **28**, 1654 (2008).
- S. Cinti *et al.*, *J. Lipid Res.* **46**, 2347 (2005).
- G. S. Hotamisligil, *Nature* **444**, 860 (2006).
- S. P. Weisberg *et al.*, *J. Clin. Invest.* **112**, 1796 (2003).
- H. Xu *et al.*, *J. Clin. Invest.* **112**, 1821 (2003).
- M. T. Veeman, J. D. Axelrod, R. T. Moon, *Dev. Cell* **5**, 367 (2003).
- H. Yamanaka *et al.*, *EMBO Rep.* **3**, 69 (2002).
- J. Hirosumi *et al.*, *Nature* **420**, 333 (2002).
- J. Y. Kim *et al.*, *J. Clin. Invest.* **117**, 2621 (2007).
- G. Solinas *et al.*, *Cell Metab.* **6**, 386 (2007).
- G. Sabio *et al.*, *Science* **322**, 1539 (2008).
- S. N. Vallerie, M. Furuhashi, R. Fucho, G. S. Hotamisligil, K. Maedler, *PLoS ONE* **3**, e3151 (2008).
- We thank S. Sono-Romanelli for technical assistance. Funded by National Institutes of Health grants (AG34972, HL86785, AG15052, and HL151587) to K.W.

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1188280/DC1
Materials and Methods
Figs. S1 to S11
References

12 February 2010; accepted 3 June 2010
Published online 17 June 2010;
10.1126/science.1188280
Include this information when citing this paper.

Muscle Dysfunction Caused by a K_{ATP} Channel Mutation in Neonatal Diabetes Is Neuronal in Origin

Rebecca H. Clark,¹ James S. McTaggart,^{1*} Richard Webster,^{2*} Roope Mannikko,^{1*} Michaela Iberl,¹ Xiu Li Sim,¹ Patrik Rorsman,³ Maïke Glitsch,¹ David Beeson,² Frances M. Ashcroft^{1†}

Gain-of-function mutations in Kir6.2 (*KCNJ11*), the pore-forming subunit of the adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channel, cause neonatal diabetes. Many patients also suffer from hypotonia (weak and flaccid muscles) and balance problems. The diabetes arises from suppressed insulin secretion by overactive K_{ATP} channels in pancreatic β -cells, but the source of the motor phenotype is unknown. By using mice carrying a human Kir6.2 mutation (Val⁵⁹→Met⁵⁹) targeted to either muscle or nerve, we show that analogous motor impairments originate in the central nervous system rather than in muscle or peripheral nerves. We also identify locomotor hyperactivity as a feature of K_{ATP} channel overactivity. These findings suggest that drugs targeted against neuronal, rather than muscle, K_{ATP} channels are needed to treat the motor deficits and that such drugs require high blood-brain barrier permeability.

Heterozygous gain-of-function mutations in the gene (*KCNJ11*) encoding the Kir6.2 subunit of the adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channel can give rise to intermediate DEND (iDEND) syndrome, a rare genetic disorder characterized by neonatal diabetes accompanied by muscle hypo-

tonia, delayed speech and motor milestones, and balance problems (1). Hypotonia (lack of muscle tone), which is usually most severe in the lower limbs, may account in part for the gait impairments. K_{ATP} channels are widely expressed (2, 3), and it is not clear whether the hypotonia in iDEND results from overactive K_{ATP} channels in muscle or in nerve. In the former case, muscle excitability would be directly reduced by excess hyperpolarizing K_{ATP} conductance, whereas in the latter neuronal regulation of muscle contraction would be compromised. To distinguish between these possibilities, we selectively expressed in mice a human gain-of-function Kir6.2 mutation in either muscle or nerve and evaluated its effect on muscle

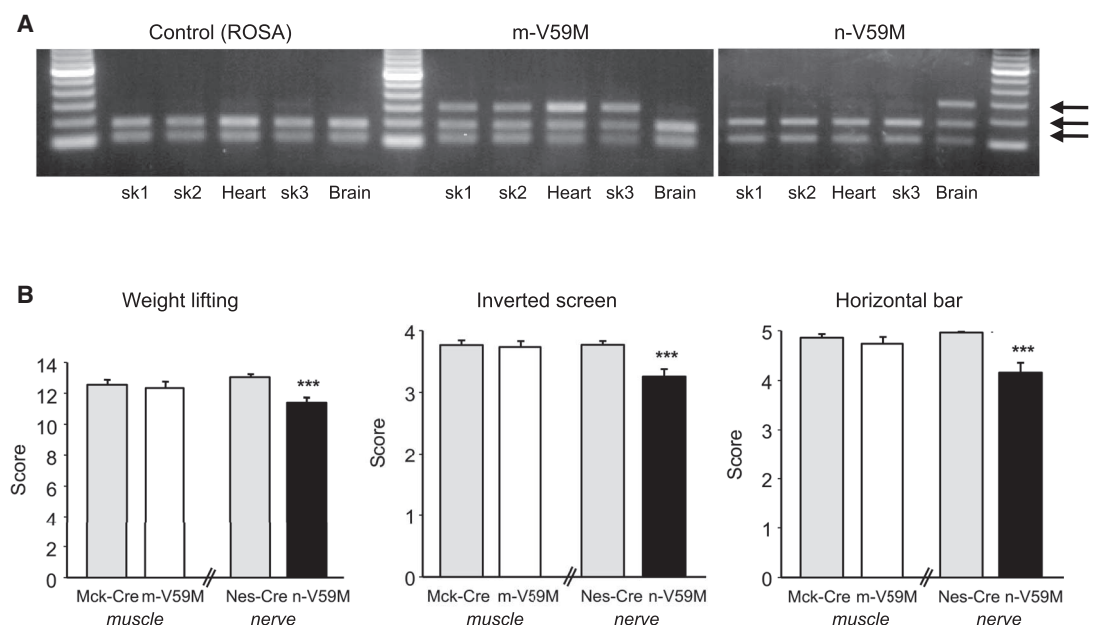
strength and motor coordination. We selected the Kir6.2-Val⁵⁹→Met⁵⁹ (V59M) mutation, which is the most common cause of iDEND (>50% of cases). Mice expressing this mutation selectively in pancreatic β -cells exhibit severe diabetes but no neurological problems (4).

We used a Cre-lox approach in combination with the muscle creatine kinase promoter (Mck-Cre mice) or the nestin promoter (Nes-Cre mice) to selectively target expression of Kir6.2-V59M to either muscle (m-V59M mice) or nerve (n-V59M mice), respectively (5). Nestin is expressed in neuronal precursor cells, and nestin-Cre is thus expected to target all neurons. However, because K_{ATP} channels comprise both Kir6.2 (pore-forming) and SUR (regulatory) subunits (3) and SUR is essential for both proper channel function and plasma membrane targeting (6), only those neurons that endogenously express SUR will express functional transgenic K_{ATP} channels. SUR1 mRNA expression was unaffected in mutant mice, suggesting that channel density will also be unaltered (fig. S1).

The analysis in Fig. 1A confirms that Kir6.2-V59M mRNA is expressed in the muscle but not the brain of m-V59M mice, in the brain but not the muscle of n-V59M mice, and not at all in control mice (5). Furthermore, endogenous wild-type and introduced mutant Kir6.2 mRNAs are expressed at comparable levels (fig. S1). This establishes our hemizygous mouse as a plausible model for human patients with the V59M mutation, all of whom are heterozygotes (1).

On three tests of muscle strength (5), mice selectively expressing the Kir6.2-V59M mutation in muscle performed equally well to control mice (Fig. 1B and fig. S2). Likewise, their motor control and balance were unaffected (5) (Fig. 2A and fig. S3). In contrast, mice in which Kir6.2-

Fig. 1. Muscle function is impaired by expression of Kir6.2-V59M in neurons but not in muscle. **(A)** Kir6.2 expression in tissue isolated from control, m-V59M, or n-V59M mice. Wild-type, but not mutant, cDNA is cut by the restriction enzyme BtsCI; two bands thus indicate the presence of the wild-type gene only, and three bands indicate both wild-type and mutant genes. sk1, quadriceps muscle; sk2, triceps muscle; sk3, diaphragm. Data are representative of experiments on four ROSA and four m-V59M mice done in parallel and three ROSA and three n-V59M mice performed in parallel [supporting online material (SOM) text and additional control data in fig. S1A]. **(B)** Weight lifting, inverted screen, and horizontal bar tests on 12-week-old m-V59M ($n = 42$), n-V59M ($n = 31$), and control mice (Mck-Cre, $n = 35$; Nes-Cre, $n = 53$). Mean $\pm$ SEM. *** $P < 0.001$ [Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks]. Additional control data in fig. S2.



V59M was targeted to neurons performed less well in all tests of muscle function: they were unable to lift weights as effectively or to hang as long from an inverted screen or a horizontal bar

(Fig. 1B and fig. S2). n-V59M mice also showed impaired balance control (Fig. 2A and fig. S3). For example, they took three times longer than controls to turn around on a thin rod suspended

above the ground, and many fell off while attempting to do so (movies S1 and S2). The n-V59M mice also fell off a rotating rod earlier than control mice.

Fig. 2. Motor coordination and locomotor activity are impaired by expression of Kir6.2-V59M in neurons but not in muscle. **(A)** (Left) Orientation time and (middle) percentage of falls on the static rod test and (right) time before falling from a rotating rod for m-V59M, n-V59M, and control (Mck-Cre, Nes-Cre) mice. Same mice as in Fig. 1. **(B and C)** Duration of time spent in spontaneous physical activity (B) or using a free-running wheel (C) over a 23-hour period for 12-week-old n-V59M ($n = 13$), Nes-Cre ($n = 14$), Rosa ($n = 14$), and wild-type (WT) ($n = 4$) littermates. Mean $\pm$ SEM. $**P < 0.01$. $***P < 0.001$ (one-way ANOVA). Additional control data are in fig. S3.

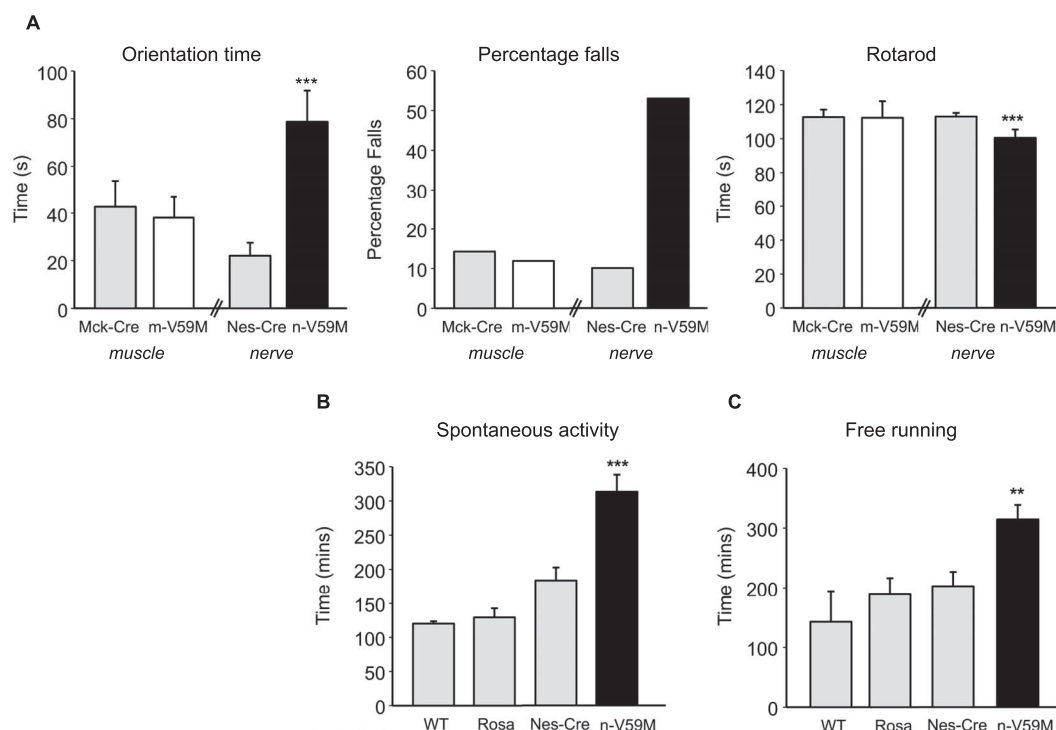
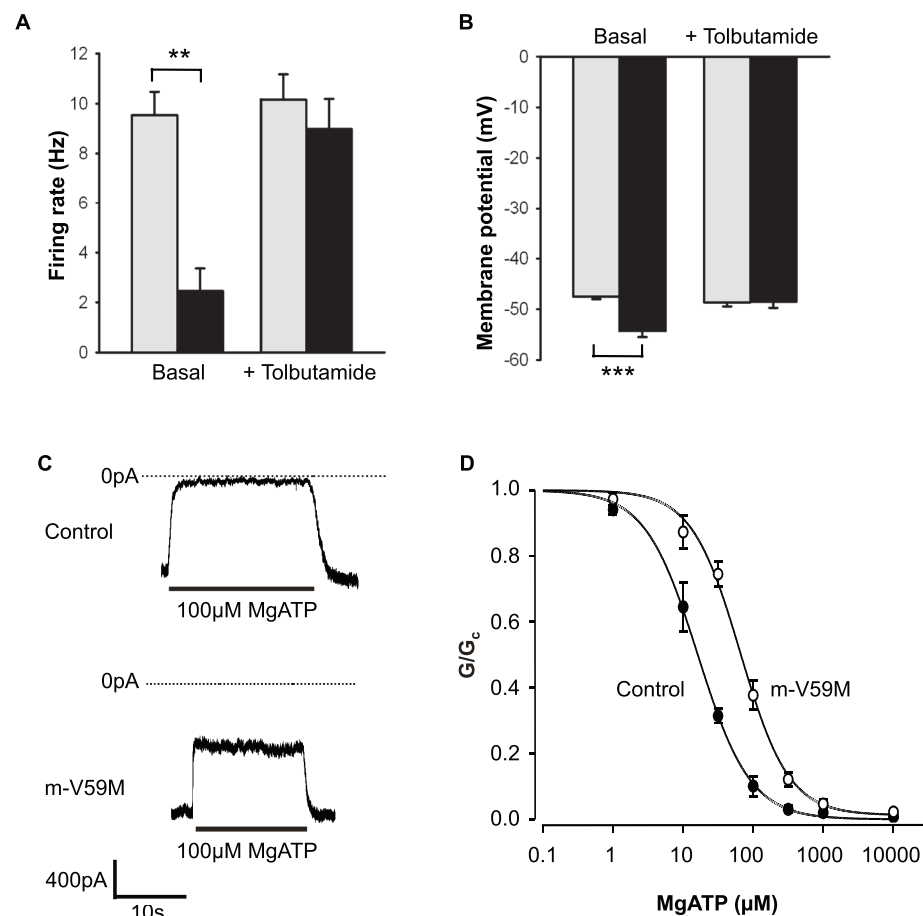


Fig. 3. K_{ATP} channel activity in Purkinje cells of n-V59M mice and skeletal muscle of m-V59M mice. **(A)** Mean ($\pm$ SEM) action potential frequency of control (gray) and n-V59M (black) neurons in the absence (for control, $n = 9$ mice, 34 neurons; for n-V59M, $n = 3$ mice, 26 neurons) and presence (for control, $n = 7$ mice, 16 neurons; for n-V59M, $n = 3$ mice, 15 neurons) of 500 μ M tolbutamide. Cell-attached recordings. $**P = 0.002$ (t test). **(B)** Mean ($\pm$ SEM) resting membrane potential of control (gray, $n = 17$ neurons, six mice) and n-V59M neurons (black, $n = 13$ neurons, five mice) in the absence and then the presence of 200 μ M tolbutamide. Whole-cell recordings. $***P < 0.001$ (t test). **(C)** K_{ATP} channel currents recorded at -60 mV in inside-out patches from control (above) or m-V59M (below) flexor digitoralis brevis (FDB) muscle. **(D)** Mean ($\pm$ SEM) MgATP sensitivity of K_{ATP} channels in inside-out patches from control ($\bullet$, $n = 4$; $IC_{50} = 15$ μ M) or m-V59M ($\circ$, $n = 4$; $IC_{50} = 66$ μ M) FDB muscle.



These results demonstrate that n-V59M mice are impaired in muscle strength, balance, and motor coordination. Their motor problems could be due either to reduced excitability of central nervous system (CNS) neurons or to decreased transmitter release at the neuromuscular junction. Studies of isolated nerve-muscle preparations (5) ruled out the latter possibility, because no differences were observed in the amplitude and frequency of miniature endplate potentials or evoked endplate potentials between control and n-V59M mice (fig. S4). There was also no difference in the muscle resting membrane potential. Similar results were found for m-V59M mice (fig. S5). This suggests that the motor difficulties experienced by the mice originate in the CNS.

The n-V59M mice also displayed hyperactivity, spontaneously moving around more frequently (Fig. 2B) and staying significantly longer than controls in free-running wheels (5) (Fig. 2C and fig. S6). Although hyperactivity is not included as a characteristic feature of iDEND

syndrome (1), several reports have indicated that once iDEND children have learned to walk they exhibit pronounced hyperactivity (7–9). Our results raise the possibility that this is caused by K_{ATP} channel overactivity and might therefore be included as a feature of iDEND.

To confirm that neurons are indeed affected by the Kir6.2-V59M mutation, we recorded the electrical activity of cerebellar Purkinje cells in acute brain slices (5). K_{ATP} channels are highly expressed in multiple brain regions, including those involved in movement (e.g., cortex and cerebellum) (10, 11), but we selected Purkinje cells because of their importance for motor control. In both cell-attached (Fig. 3, A and B, and fig. S7) and whole-cell (fig. S7C) recordings, action potential frequency was substantially lower in Purkinje cells of n-V59M mice than of controls. Tolbutamide, a specific inhibitor of K_{ATP} channels, increased the firing rate of n-V59M cells to that of control neurons but had no effect on control cells (Fig. 3A). The resting membrane potential

of n-V59M Purkinje cells was also more hyperpolarized than in controls and was restored by tolbutamide (Fig. 3B). This suggests that, in n-V59M mice, an increased K_{ATP} current in Purkinje cells suppresses firing and may thereby impair motor function. Neurons other than Purkinje cells are also likely to contribute to the motor phenotype.

Patch-clamp recordings of isolated muscle fibers from m-V59M mice revealed that the ATP sensitivity of the native muscle K_{ATP} channel is reduced compared with that of control mice (Fig. 3, C and D, and table S1). Why should the Kir6.2-V59M mutation fail to affect the muscle membrane potential, yet hyperpolarize neurons? We reasoned this might reflect differences in tissue metabolism (and thus intracellular ATP concentrations) or, alternatively, that it may be due to the different SUR isoforms that compose muscle (SUR2A) and nerve (SUR1) K_{ATP} channels (3). To distinguish between these possibilities, we compared the responses of muscle and neuronal types of K_{ATP} channel heterologously expressed in the

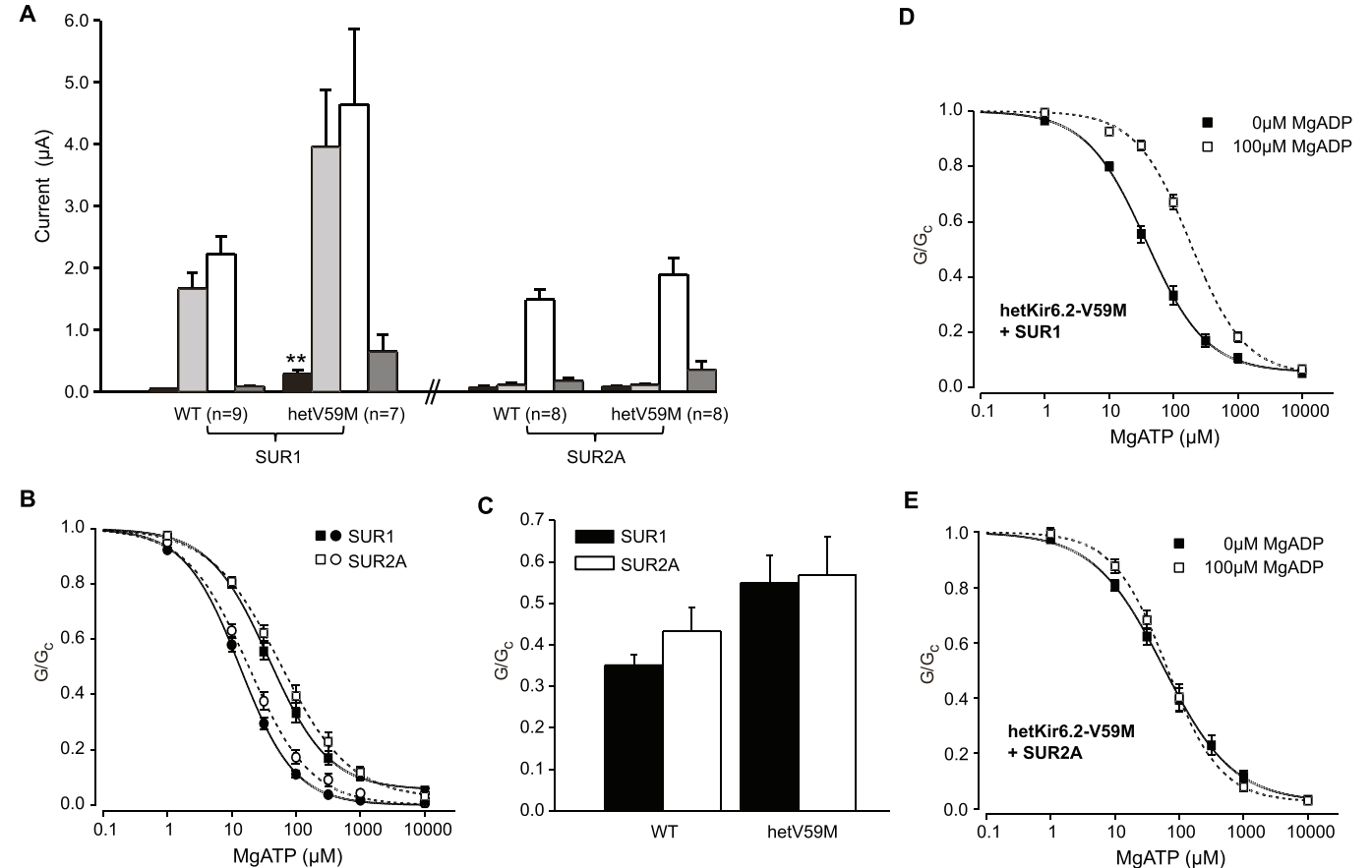


Fig. 4. Kir6.2/SUR1 and Kir6.2/SUR2A show different sensitivities to metabolic inhibition. (A) Mean ($\pm$ SEM) steady-state whole-cell K_{ATP} currents evoked by a voltage step from -10 to -30 mV before (black) and after (pale gray) application of 3 mM azide for oocytes injected with Kir6.2 (WT) or a 1:1 mixture of Kir6.2 and Kir6.2-V59M (hetV59M) mRNA, plus either SUR1 or SUR2A mRNAs. White, 3 mM azide plus 300 μ M diazoxide (SUR1) or 100 μ M pinacidil (SUR2A). Dark-gray, 3 mM azide, 300 μ M diazoxide, and 0.5 mM tolbutamide (SUR1) or 3 mM azide, 100 μ M pinacidil, and 10 μ M glibenclamide (SUR2A). The number of oocytes is indicated. $**P < 0.001$ with respect to WT (t test). (B) Mean ($\pm$ SEM) MgATP sensitivity of Kir6.2/SUR1 ($\bullet$, $n = 15$), Kir6.2/SUR2A ($\circ$, $n = 15$), hetKir6.2-V59M/SUR1 ($\blacksquare$,

$n = 12$), and hetKir6.2-V59M/SUR2A ($\square$, $n = 16$) channels. K_{ATP} conductance (G) is expressed relative to that in the absence of nucleotides (G_c). The curves are the best fit of the Hill equation to the mean data (SOM text and table S1). (C) MgADP activation of channels composed of WT Kir6.2 (left) or Kir6.2-V59M (right) plus either SUR1 (black) or SUR2A (white). Increase in the K_{ATP} conductance in the presence of 100 μ M MgADP. Conductance (G) is expressed as a fraction of the maximal conductance in the absence of MgADP (G_c). Mean $\pm$ SEM. (D and E) Mean ($\pm$ SEM) MgATP sensitivity of hetKir6.2-V59M/SUR1 (D) and hetKir6.2-V59M/SUR2A (E) channels in the absence ($\blacksquare$; SUR1, $n = 12$; SUR2A, $n = 16$) or presence ($\square$; SUR1, $n = 14$; SUR2A, $n = 7$) of 100 μ M MgADP (SOM text and table S2).

same cell type (*Xenopus* oocytes) (5). Channels composed of wild-type Kir6.2 and either SUR1 or SUR2A were closed under resting conditions because of the high oocyte intracellular ATP concentration. Lowering intracellular ATP concentration by metabolic inhibition activated large whole-cell Kir6.2/SUR1 currents but had no effect on Kir6.2/SUR2A channels (Fig. 4A). The Kir6.2-V59M mutation enhanced the resting Kir6.2/SUR1 current but had no effect on Kir6.2/SUR2A currents, which remained closed even when metabolism was inhibited. Thus, differences in SUR isoforms explain why muscle is unaffected by the Kir6.2-V59M mutation, whereas neuronal electrical activity is reduced.

Adenine nucleotides inhibit K_{ATP} channels by binding to Kir6.2 and (when Mg^{2+} is present) activate them via interaction with SUR (12, 13). $MgATP$ block (Fig. 4B) and magnesium adenosine diphosphate ($MgADP$) activation in the absence of $MgATP$ (Fig. 4C) were similar for Kir6.2/SUR1 and Kir6.2/SUR2A channels whether wild-type or mutant. By contrast, whereas $MgADP$ activated SUR1-containing channels when $MgATP$ was present, markedly shifting the ATP dose-response curve (Fig. 4D), this was not the case for SUR2A-containing channels (Fig. 4E). This difference may underlie the different sensitivities of mutant Kir6.2/SUR1 and Kir6.2/SUR2A channels to metabolic inhibition.

Our results demonstrate that hemizygous mice carrying the Kir6.2-V59M mutation provide a faithful model for the muscle weakness, balance problems, and hyperactivity seen in iDEND children. They also confirm that analogous problems in human patients are not a secondary consequence of diabetes (or of insulin-induced hypoglycemia), because the mice have normal blood glucose levels (fig. S8). Importantly, the data indicate that the motor deficits result from impairment of neuronal, not muscle, function.

Over the past 4 years, sulphonylurea therapy has become the treatment of choice for neonatal diabetes and iDEND (14). These drugs stimulate insulin secretion by blocking open K_{ATP} channels (15). They also improve the neurological problems in many (but not all) iDEND patients. Some children begin to walk or talk for the first time shortly after starting

therapy (16); in most cases, hypotonia and fine motor control are improved (17) and hyperactivity is reduced (9, 10). Our data suggest that these improvements reflect sulphonylurea block of inappropriately active K_{ATP} channels in brain neurons.

Most iDEND patients are currently treated with glibenclamide, which interacts with both SUR1 and SUR2A. Our hypothesis that their muscle problems are likely neuronal in origin means that it may also be possible to use SUR1-specific sulphonylureas such as tolbutamide, glizide, or nateglinide (15). Indeed, there is one report that glizide can enhance movement control in a patient (17).

The choice of sulphonylurea used for type 2 diabetes therapy has long been debated (18–20). In particular, there has been concern that these drugs may interact adversely with cardiac (SUR2A-containing) K_{ATP} channels. K_{ATP} channel activation plays an important role in cardiac ischemic preconditioning in the heart (3), and this effect is prevented by glibenclamide (21). Neonatal diabetes patients require higher sulphonylurea doses than type 2 diabetes patients, and they may be expected to take the drugs over a much longer time period. Our finding that the motor problems in iDEND are neuronal in origin suggests that it is unnecessary to use blockers of muscle (SUR2A-containing) K_{ATP} channels to ameliorate the motor problems. Thus, it may be worth considering whether iDEND patients would be better treated with SUR1-specific drugs to avoid potential cardiac cross-reactivity.

Lastly, when examined in the light of our mouse data, the success of sulphonylureas in treating the muscle impairments of iDEND children suggests that these drugs gain access to the brain. This means that efforts to improve sulphonylurea therapy for motor problems in iDEND patients should focus on identifying those that have high blood-brain barrier permeability.

References and Notes

1. A. T. Hattersley, F. M. Ashcroft, *Diabetes* **54**, 2503 (2005).
2. F. M. Ashcroft, *Am. J. Physiol.* **293**, E880 (2007).
3. C. G. Nichols, *Nature* **440**, 470 (2006).
4. C. A. Girard *et al.*, *J. Clin. Invest.* **119**, 80 (2009).

5. Materials and methods are available as supporting material on Science Online.
6. N. Zerangue, B. Schwappach, Y. N. Jan, L. Y. Jan, *Neuron* **22**, 537 (1999).
7. J. V. Sagen *et al.*, *Diabetes* **53**, 2713 (2004).
8. A. S. Slingerland *et al.*, *Diabet. Med.* **25**, 277 (2008).
9. W. Mlynarski *et al.*, *Nat. Clin. Pract. Neurol.* **3**, 640 (2007).
10. C. Karschin, C. Ecke, F. M. Ashcroft, A. Karschin, *FEBS Lett.* **401**, 59 (1997).
11. C. Mourre, C. Widmann, M. Lazdunski, *Brain Res.* **519**, 29 (1990).
12. S. J. Tucker, F. M. Gribble, C. Zhao, S. Trapp, F. M. Ashcroft, *Nature* **387**, 179 (1997).
13. C. G. Nichols *et al.*, *Science* **272**, 1785 (1996).
14. E. R. Pearson *et al.*, *N. Engl. J. Med.* **355**, 467 (2006).
15. F. M. Gribble, F. Reimann, *Diabetologia* **46**, 875 (2003).
16. A. S. Slingerland, R. Nuboer, M. Hadders-Algra, A. T. Hattersley, G. J. Bruining, *Diabetologia* **49**, 2559 (2006).
17. J. C. Koster *et al.*, *J. Clin. Endocrinol. Metab.* **93**, 1054 (2008).
18. U. Quast, D. Stephan, S. Bieger, U. Russ, *Diabetes* **53** (suppl.), S156 (2004).
19. S. H. Simpson, S. R. Majumdar, R. T. Tsuyuki, D. T. Eurich, J. A. Johnson, *Can. Med. Assoc. J.* **174**, 169 (2006).
20. UK Prospective Diabetes Study (UKPDS) Group, *Lancet* **352**, 837 (1998).
21. S. Ghosh, N. B. Standen, M. Galiñanes, *J. Am. Coll. Cardiol.* **37**, 711 (2001).
22. We thank C. Girard for generating the ROSA mouse and help with early experiments; J. Brüning for the Mck-Cre mice; R. Deacon for help with the mouse behavioral tests and for the use of the OXION mouse behavior facility; and the Wellcome Trust, the Medical Research Council, the Royal Society, the European Union (LHSB-CT-2004-005137 and HEALTH-F4-2007-201924), the Muscular Dystrophy Campaign, and Myasthenia Gravis Association for support. R.H.C. holds an OXION Wellcome Trust prize studentship and J.S.M. holds a Medical Research Council studentship. F.M.A. is a Royal Society University Research Professor.

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1186146/DC1
Materials and Methods
SOM Text
Figs. S1 to S10
Tables S1 to S4
Movies S1 and S2

18 December 2009; accepted 11 June 2010
Published online 1 July 2010;
10.1126/science.1186146
Include this information when citing this paper.

NEW PRODUCTS: INTERACTOMICS

MOLECULAR INTERACTION ANALYSIS



GE Healthcare/Biacore

For info: 800-526-3593 | www.biacore.com

The Biacore 4000 is a powerful solution for large-scale, label-free molecular interaction analysis in drug discovery, from early screening to characterization. The system delivers high-quality binding, kinetic, affinity, concentration, and specificity data in both screening assays and detailed characterization studies. Designed for large-scale parallel interaction analyses, the Biacore 4000 is capable of analyzing up to 4,800 interactions in 24 hours. Dedicated software packages are available to support small-molecule drug discovery and antibody screening and characterization. In combination with the LMW Extension Package software, the Biacore 4000 delivers the sensitivity, throughput, and high-quality data required for fragment screening, lead selection, and optimization. The Antibody Extension Package provides dedicated software tools for high throughput kinetic studies and epitope mapping, enabling rapid identification of promising candidates.

FUNCTIONAL PROTEIN EXPRESSION KIT

The Invitrogen MembranePro Functional Protein Expression (FPE) Kit is an easy-to-use method for obtaining functional membrane proteins, including GPCRs, from mammalian cells. The kit can be used to clone and transfect GPCRs or other membrane protein genes into 293FT cells, collect the culture media after 48 hours, and precipitate the secreted membrane particles overnight. After resuspension, the particles are ready for immediate use in downstream biochemical studies. The simple process produces particles comparable to conventional membrane preparations with the added advantage of increased receptor density. MembranePro products are offered in two configurations, an expression kit with 10 reactions and a support kit available in 10, 60, or 600 reaction sizes.

Life Technologies Corporation

For info: 760-603-7200 | www.lifetechnologies.com

CHIP-CHIP KITS

Magna ChIP² chromatin immunoprecipitation DNA microarray (ChIP-chip) kits allow users to map entire gene regulatory networks and patterns of epigenetic marks using microarray technology. The kits provide reagents, microarrays, and validated protocols for the entire ChIP-chip workflow, allowing users to examine protein-DNA interactions on a genome-wide scale. Two types of kits are available to accommodate different scientific approaches. The Magna ChIP² Promoter Microarray kits contain the necessary reagents for ChIP-chip and either human or mouse Agilent promoter microarrays. The Magna ChIP² Universal Microarray kits supply the necessary reagents for performing ChIP-chip with microarrays provided by the user.

Millipore

For info: 800-645-5476 | www.millipore.com/chip2chip

HUMAN MIRNA EXPRESSION ASSAY KIT

The Human microRNA (miRNA) Expression Assay Kit provides a simple and precise way to profile the human miRNA transcriptome in a single tube. Users can perform highly multiplexed, direct digital detection, as well as counting of miRNAs at single-base resolution without the need for polymerase chain reaction (PCR) amplification. The comprehensive and cost-effective assay enables users to profile more than 700 human and human-viral miRNAs with a specificity and sensitivity comparable to quantitative PCR. The assay kit contains all of the reagents and consumables required to conduct miRNA and gene expression experiments and can be combined with the easy-to-use, fully automated target profiling nCounter Analysis System.

NanoString Technologies

For info: 888-358-6266 | www.nanostring.com

COATED 96-WELL PLATES

Well-Coated plates are ready-to-use coated plates that are suitable for colorimetric, chemiluminescence, and fluorescent detection systems. The plates are supplied pre-blocked in G-Biosciences Superior Blocking Buffer and are available as single 96-well plates or as 12 x 8-well strips in a 96-well holder in clear, white, or black polystyrene. Well-Coated plates are offered with the following coatings: protein A, protein G, protein A/G, protein L, goat anti-mouse antibody or goat anti-rabbit antibody for binding antibodies; neutravidin, streptavidin and biotin for biotin studies; nickel and glutathione for His or GST tagged recombinant protein binding; and activated plates for binding proteins and other molecules through amine or sulfhydryl residues.

G-Biosciences

For info: 314-991-6034 | www.GBiosciences.com

Electronically submit your new product description or product literature information! Go to www.sciencemag.org/products/newproducts.dtl for more information. Newly offered instrumentation, apparatus, and laboratory materials of interest to researchers in all disciplines in academic, industrial, and governmental organizations are featured in this space. Emphasis is given to purpose, chief characteristics, and availability of products and materials. Endorsement by *Science* or AAAS of any products or materials mentioned is not implied. Additional information may be obtained from the manufacturer or supplier.